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Remarks

Claims 1-6, 10, 13-21 are pending. New claim 21 has been added. Claims 7, 9, 11, and 12, which had been withdrawn by the Examiner, have been canceled because they read on a non-elected invention. Claim 8 has been canceled because it was rendered redundant by the amendments to claim 1. Claims 1, 10, 14, 19 and 20 have been amended.

Support for the claim amendments and new claim 21 can be found throughout the application, including the claims as originally filed. Importantly, no new matter has been added to the claims. Further, the amendments to the claims should not be construed to be an acquiescence to any of the rejections. The amendments to the claims are being made solely to expedite the prosecution of the above-identified application. Applicant expressly reserves the option to prosecute further the same or similar claims in the instant application or subsequent patent applications entitled to the priority date of the instant application. 35 USC § 120.

Election/Restrictions

As per the telephonic interview conducted on March 29, 2004, the Applicants hereby affirm the election of invention I. Accordingly, claims 1-6, 8, 10 and 13-20 have been canceled or redrawn to compounds of formula (I) where X represents -N(R_x)-, pharmaceutical compositions containing these compounds and methods of using these compounds.

Specification

On August, 20, 2003 the US Patent & Trademark Office Mail Room acknowledged the receipt of this application, including a specification section of 42 pages. The abstract was found on page 42 of the specification. An abstract is provided; please note that this does *not* constitute the introduction of new matter because the abstract is a replacement of the original.

Response to Rejections under 35 U.S.C. 112¶1

Claim 14 stands rejected under 35 USC 112¶1, based on the Examiner's contention that the Specification does not reasonably provide enablement for "preventing" a pathological condition in a mammal which is associated with abnormal activity of a metabotropic glutamate receptor. The Applicants respectfully concede that certain pathological conditions in mammals are due to a constellation of physiological abnormalities. Additionally, the Applicants concede that a pathological condition in a mammal which is not due solely to an abnormality in the activity of a metabotropic glutamate receptor may not be "prevented" by the administration of a compound which only modulates the activity of a metabotropic glutamate receptor. Accordingly, the Applicants have amended claim 14 to cover only "treating a pathological condition or symptom in a mammal which is associated with abnormal activity of a metabotropic glutamate receptor...."

However, to more completely claim the scope of the invention for which the Applicants are entitled to a right to exclude, the Applicants have added new claim 21, directed to a method of "preventing" a pathological condition or symptom in a mammal "caused by" an abnormality in the activity of a metabotropic glutamate receptor. The Applicants respectfully contend that new claim 21 is enabled by the Specification because one of ordinary skill in the arts of medicinal chemistry and pharmacology would conclude, based on the data contained in the Specification, that the subject compounds are capable of modulating the activity of metabotropic glutamate receptors. Consequently, one of ordinary skill in the arts of medicinal chemistry and pharmacology would also conclude that the subject compounds could be used to prevent a pathological condition or symptom in a mammal caused by abnormal activity of a metabotropic glutamate receptor.

In addition, claims 14-19 stand rejected under 35 U.S.C. 112¶1, based on the Examiner's contention that the claimed subject matter was not described in the Specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention because the

Specification does not contain any teaching or guidance that glutamate occurs abnormally in the disorders recited in the rejected claims.

Therefore, the Applicants respectfully submit herewith prior-art publications, **Exhibits F-AC**, and present below synopses thereof highlighting the support each Exhibit provides for pathological conditions comprised by the rejected claims. Further, the Applicants respectfully draw the Examiner's attention to the fact that all of the publications submitted as Exhibits were published prior to the instant application's earliest priority date, July 17, 1997. Based on the teachings of Exhibits F-AC and the fact that all of the disorders recited in the rejected claims have a neurological component, the Applicants respectfully contend that before the earliest priority date claimed in the instant application one of ordinary skill in the art of pharmacology would have known that glutamate abnormality occurs in all of the disorders recited in the rejected claims. Consequently, the Applicants respectfully request the withdrawal of the Examiner's rejection of claims 15-19 under 35 USC 112¶1.

Exhibit F: Rothstein, J.D. et al. "Excitatory Amino Acid Metabolism in Amyotrophic Lateral Sclerosis" *Annals of Neurology* **1990**, *28*, 18-25. Exhibit F teaches that patients with Amyotrophic Lateral Sclerosis (ALS) have abnormal levels of excitatory amino acids in their cerebrospinal fluid. Further, it teaches that these abnormalities likely play a role in the neurodegeneration associated with ALS. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating ALS in a mammal.

Exhibit G: Siliprandi, R. et al. "Activation of the Glutamate Metabotropic Receptor Protects Retina Against *N*-Methyl-D-Aspartate Toxicity" *European Journal of Pharmacology* **1992**, *219*, 173-174. Exhibit G teaches that intraocular administration of a metabotropic glutamate receptor agonist reduced retinal damage in rats caused by the excitatory amino acid N-methyl-D-aspartate. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a

therapeutic method for treating ocular damage associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit H: Bruno, V. et al. "Protective Effect of the Metabotropic Glutamate Receptor Agonist, DCG-IV, Against Excitotoxic Neuronal Death" *European Journal of Pharmacology* **1994**, *256*, 109-112. Exhibit H teaches that an agonist of two subtypes of metabotropic glutamate receptors protects cultured neurons against damage caused by excitatory amino acids. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit I: Bruno, V. et al. "Activation of Group III Metabotropic Glutamate Receptors is Neuroprotective in Cortical Cultures" *European Journal of Pharmacology* **1996**, *310*, 61-66. Exhibit I teaches that the activation of group III metabotropic glutamate receptors protects neurons against excitotoxic neuronal death. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit J: Lombardi, G. et al. "Glutamate Receptor Antagonists Protect Against Ischemia-Induced Retinal Damage" *European Journal of Pharmacology* **1994**, *27*, 489-495. Exhibit J teaches that excitatory amino acids are involved in ischemia-induced neuronal death in the retina. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating ocular damage, and neuronal damage due to hypoxia and ischemia in general, associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit K: Bruno, V. et al. "Activation of Class II or III Metabotropic Glutamate Receptors Protects Cultured Cortical Neurons Against Excitotoxic Degeneration"

European Journal of Neuroscience **1995**, *7*, 1906-1913. Exhibit K teaches that agonists of class II or III metabotropic glutamate receptors are highly potent and efficacious in protecting cultured cortical neurons against toxicity induced by excitatory amino acids. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit L: Choi, D.W. "Calcium-Mediated Neurotoxicity: Relationship to Specific Channel Types and Role in Ischemic Damage" *Trends in Neuroscience* **1988**, *11*, 465-468. Exhibit L teaches that the neuronal injury associated with hypoxia and ischemia is linked to excessive activation of glutamate receptors. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage in hypoxia and ischemia associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit M: Koroshetz, W.J. et al. "Emerging Treatments for Stroke in Humans" *Trends in Pharmacological Science* **1996**, *17*, 227-233. Exhibit M teaches that a number of drugs which act at glutamate receptors limit the size of cerebral infarcts in animal models of ischemia. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage in ischemia in a mammal.

Exhibit N: Lipton, S.A. "Models of Neuronal Injury in AIDS: Another Role for the NMDA Receptor?" *Trends in Neurosciences* **1992**, *15*, 75-79. Exhibit N teaches that excess excitatory amino acids may be responsible for the neuronal damage suffered by the majority of AIDS patients. Further, the Exhibit teaches that antagonists of excitatory amino acid receptors attenuate the neuronal damage caused by a glycoprotein produced by HIV. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian

metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage associated with AIDS in a mammal.

Exhibit O: Nicoletti, F. et al. "Metabotropic Glutamate Receptors: A New Target for the Therapy of Neurodegenerative Disorders?" *Trends in Neurosciences* **1996**, *19*, 267-271. Exhibit O teaches that certain metabotropic glutamate receptor subtypes exert inhibitory effects on neurodegenerative processes. Further, the Exhibit teaches that metabotropic glutamate receptors are promising drug targets for the therapy of acute and/or chronic neurodegenerative diseases in mammals. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit P: Riedel, G. "Function of Metabotropic Glutamate Receptors in Learning and Memory" *Trends in Neurosciences* **1996**, *19*, 219-224. Exhibit P teaches that pre-training treatment of a mammal with an agonist or antagonist of a metabotropic glutamate receptor blocks memory formation. Conversely, the Exhibit reasons that post-training treatment of a mammal with an agonist of a metabotropic glutamate receptor will amplify memory formation. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating cognitive deterioration, e.g., senile dementia, associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit Q: Koh, J.Y. et al. "Activation of the Metabotropic Glutamate Receptor Attenuates *N*-Methyl-D-Aspartate Neurotoxicity in Cortical Cultures" *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9431-9435. Exhibit Q teaches that a selective agonist for a metabotropic glutamate receptor attenuates neuronal damage caused by an excitatory amino acid in murine cortical cultures. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a

therapeutic method for treating neurodegeneration associated with abnormal activity of a metabotropic glutamate receptor in a mammal.

Exhibit R: Rothstein, J.D. et al. "Chronic Inhibition of Glutamate Uptake Produces a Model of Slow Neurotoxicity" *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6591-6595. Exhibit R teaches that persistent exposure of neurons to elevated synaptic concentrations of glutamate causes gradual neurodegeneration. Further, the Exhibit teaches that non-NMDA antagonists, i.e., antagonists of metabotropic glutamate receptors as opposed to antagonists of ionotropic glutamate receptors, protect neurons from the neurodegeneration associated with persistent exposure of neurons to elevated synaptic concentrations of glutamate. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal concentrations of glutamate in a mammal.

Exhibit S: Birrell, G.J. et al. "(1S,3R)-1-Aminocyclopentane-1,3-Dicarboxylic Acid Attenuates *N*-Methyl-D-Aspartate-Induced Neuronal Cell Death in Cortical Cultures via a Reduction in Delayed Ca^{2+} Accumulation" *Neuropharmacology* **1993**, *32*, 1351-1358. Exhibit S teaches that an agonist of metabotropic glutamate receptors attenuates neural cell death caused by exposure to an excitatory amino acid. Further, the Exhibit teaches that the same agonist alone is not neurotoxic. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal concentrations of excitatory amino acids in a mammal.

Exhibit T: Ambrosini, A. et al. "Metabotropic Glutamate Receptors Negatively Coupled to Adenylate Cyclase Inhibit *N*-Methyl-D-Aspartate Receptor Activity and Prevent Neurotoxicity in Mesencephalic Neurons *In Vitro*" *Molecular Pharmacology* **1995**, *47*, 1057-1064. Exhibit T teaches that metabotropic glutamate receptors are present in mouse mesencephalic (midbrain) neurons. Additionally, the Exhibit teaches

that pretreatment of mouse mesencephalic (midbrain) neurons with an agonist of metabotropic glutamate receptors inhibits, in a dose-dependent fashion, the neurodegenerative effects of an excitatory amino acid, NMDA. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with abnormal concentrations of excitatory amino acids in a mammal.

Exhibit U: Copani, A. et al. "Activation of Metabotropic Glutamate Receptors Protects Cultured Neurons Against Apoptosis Induced by b-Amyloid Peptide" *Molecular Pharmacology* **1995**, *47*, 890-897. Exhibit U teaches that prolonged exposure of cultured neural cells to a fragment of the b-amyloid peptide results in apoptosis, programmed cell death. Further, the Exhibit teaches that a selective agonist of metabotropic glutamate receptors substantially attenuates the apoptosis induced by the aforementioned fragment of the b-amyloid peptide. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neurodegeneration associated with Alzheimer's disease in a mammal.

Exhibit V: Fundytus, M.E. et al. "Effect of Activity at Metabotropic, as well as Ionotropic (NMDA), Glutamate Receptors on Morphine Dependence" *British Journal of Pharmacology* **1994**, *113*, 1215-1220. Exhibit V teaches that selective antagonists of metabotropic glutamate receptors significantly attenuated withdrawal symptoms in rats addicted to morphine. Therefore, because withdrawal symptoms substantially contribute to the inability to stop using a drug in addicted mammals, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating drug addiction in a mammal.

Exhibit W: McCulloch, J. "Excitatory Amino Acid Antagonists and Their Potential for the Treatment of Ischaemic Brain Damage in Man" *British Journal of Clinical Pharmacology* **1992**, *34*, 106-114. Exhibit W teaches that the massive increase

in the extracellular concentration of glutamate that accompanies cerebral ischemia contributes to the neuronal death associated with ischemia. Further, the Exhibit teaches that antagonists of receptors for excitatory amino acids markedly reduce the amount irreversible neurodegeneration accompanying ischemic insult in animal models. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage in ischemia in a mammal.

Exhibit X: Chiamulera, C. et al. "Activation of Metabotropic Receptors has a Neuroprotective Effect in a Rodent Model of Focal Ischaemia" *European Journal of Pharmacology* **1992**, *216*, 335-336. Exhibit X teaches that an agonist of metabotropic glutamate receptors significantly reduces the size of the cerebral infarct resulting from ischemic insult in mice. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage in ischemia in a mammal.

Exhibit Y: Rothstein, J.D. et al. "Neuroprotective Strategies in a Model of Chronic Glutamate-Mediated Motor Neuron Toxicity" *Journal of Neurochemistry* **1995**, *65*, 643-651. Exhibit Y teaches that drugs that alter glutamate neurotransmission, e.g., drugs that act at metabotropic glutamate receptors, are neuroprotective against slow glutamate-mediated neurotoxicity. This type of neurotoxicity is believed to be a component of ALS. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage in ischemia in a mammal.

Exhibit Z: Maiese, K. et al. "Cellular Mechanisms of Protection by Metabotropic Glutamate Receptors During Anoxia and Nitric Oxide Toxicity" *Journal of Neurochemistry* **1996**, *66*, 2419-2428. Exhibit Z teaches that agonists of metabotropic glutamate receptors both protect neuronal cells from death caused by anoxia and nitric

oxide, and attenuate the neurodegeneration caused by anoxia and nitric oxide poisoning. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage caused by anoxia or nitric oxide poisoning in a mammal.

Exhibit AA: Buisson, A. et al. "The Inhibitory mGluR Agonist, s-4-Carboxy-3-Hydroxy-Phenylglycine Selectively Attenuates NMDA Neurotoxicity and Oxygen-Glucose Deprivation-Induced Neuronal Death" *Neuropharmacology* **1995**, *34*, 1081-1087. Exhibit AA teaches that an agonist of metabotropic glutamate receptors attenuates slowly-triggered murine neuronal death caused by excitatory amino acids. Further, the Exhibit teaches that the same agonist attenuates murine neuronal cell death induced by combined oxygen/glucose deprivation. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage caused by excitatory amino acids or combined oxygen/glucose deprivation in a mammal.

Exhibit AB: Opitz, T. et al. "The Metabotropic Glutamate Receptor Antagonist (+)-a-Methyl-4-Carboxyphenylglycine Protects Hippocampal CA1 Neurons of the Rat from *In Vitro* Hypoxia/Hypoglycemia" *Neuropharmacology* **1994**, *33*, 715-717. Exhibit AB teaches that an antagonist of metabotropic glutamate receptors protected rat neurons from hypoxia/hypoglycemia-induced neurodegeneration. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating neuronal damage caused by oxygen/glucose deprivation in a mammal.

Exhibit AC: Iwasaki, Y. et al. "Plasma Amino Acid Levels in Patients with Amyotrophic Lateral Sclerosis" *Journal of the Neurological Sciences* **1992**, *107*, 219-222. Exhibit AC teaches that ALS patients have statistically significant elevations in plasma levels of excitatory amino acids, e.g., glutamate. Therefore, one of ordinary skill in the art of pharmacology would expect the compounds of the present invention, which

are agonists or antagonists of mammalian metabotropic glutamate receptors, to be useful in a therapeutic method for treating ALS in a mammal.

Response to Rejections under 35 U.S.C. 112¶2

Claim 1 stands rejected under 35 U.S.C. 112¶2 based on the Examiner's contention that the term "prodrug" is indefinite since types of prodrugs and a process for preparing them are not defined. The Applicants disagree and direct the Examiner to the Specification (page 17, lines 1-5):

> The term "prodrug" is well known in the pharmaceutical arts. As used herein, the term includes compounds that are converted *in vivo* to a compound of formula I or II, or a salt thereof. The prodrugs of the invention include derivatives that are known in the art to function as prodrugs, *e.g.*, esters or an acid of formula I or II.

The Applicants contend that this passage, in addition to the overwhelming occurrence of prodrugs in the literature (*e.g.*, Roche, E. B. (Ed.), *Design of Biopharmaceutical Properties Through Prodrugs and Analogs*, Amer Pharmaceutical Assn (MacK), first published in 1977 and now on its 7th volume), makes the term "prodrug" definite. The Applicants also wish to remark that this wording has appeared before in other members of this patent family wherein it has always been found to be not indefinite. Accordingly, the Applicants request withdrawal of the rejection.

Claim 14 stands rejected under 35 U.S.C. 112¶2 based on the Examiner's contention that the terms "a pathological condition or symptom" and "abnormal activity" are indefinite. The Applicants disagree. Please note that the specification (page 2, lines 10-20) clearly defines "abnormal activity" as including "an increase or decrease in activation of the receptors compared to normal function in said mammal." In other words, this term is defined to encompase hyperactivity and hypoactivity of any mGluR receptors. Furthermore, the Applicant argues that the term "pathological condition" is also understood through its common definition and its dependence upon "abnormal activity," as described in the Specification ("a pathological condition or symptom in a mammal which is *associated* with abnormal activity"). In addition, as was true for the

term "prodrug," the Applicants also wish to remark that this wording has appeared before in other members of this patent family wherein it has always been found to be not indefinite. Accordingly, the Applicants request withdrawal of the rejection.

Claim 19 stands rejected under 35 U.S.C. 112¶2 based on the Examiner's contention that the term "manage an addiction" is indefinite since it is not clear to what type of addiction reference is made. The Applicants have amended claim 19 to depend upon claim 14, and to claim a method of treating a condition or symptom associated with morphine dependence. Accordingly, the Applicants request withdrawal of the rejection.

Claim 20 stands rejected under 35 U.S.C. 112¶2 based on the Examiner's contention that the term "detectable label" is indefinite since the types of isotopes used as well as a process for preparing specific labeled compounds are not defined. Therefore, the Applicants have amended claim 20 to include a Markush group wherein the detectable label is selected from the group consisting of ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{13}N, ^{15}N, and ^{18}O. Support for this amendment can be found in the Specification on page 23, lines 10-14. As to the method of preparing such labeled compounds, this application explicitly teaches the synthesis of unlabeled compounds to be used as bicyclic metabotropic glutamate receptor ligands. However, all that is needed for one skilled in the art to synthesize an isotopically labeled compound is to replace a "cold" reagent (*i.e.*, a reagent that is not radioactive) with an isotopically-labeled variant; in selecting the reagent one might consult a general reference text such as the "Synthesis and Applications of Isotopically Labeled Compounds," which was published by Wiley in 1997. Accordingly, the Applicants request reconsideration of amended claim 20.

Claim Objections

Claims 1-6, 8, 10 and 13-20 are objected to based on the Examiner's contention that they contain non-elected subject matter. As mentioned above, claim 8 has been canceled because it was rendered redundant by the amendments to claim 1. In the remaining claims, their subject matter has been amended to contain only elected subject matter. Accordingly, the Applicants request withdrawal of the objection to amended claims 1-6, 10 and 13-20.

Allowable Subject Matter

The Applicants gratefully acknowledge the Examiner's determination that the instant compounds are neither disclosed or obvious in view of the prior art and thus constitute allowable subject matter.

Fees

The Applicants believe no fee is due in connection with the filing of this paper. Nevertheless, the Director is hereby authorized to charge any required fee to our Deposit Account, **06-1448**.

Conclusion

In view of the above amendments and remarks, it is believed that the pending claims are in condition for allowance. If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is urged to contact the undersigned at (617) 832-1000.

Respectfully submitted,
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Abstract

The present invention provides bicyclic metabotropic glutamate receptor ligands, as well as compositions comprising such ligands, and methods for their use.

Exhibit F (10/644,645)

Abnormal Excitatory Amino Acid Metabolism in Amyotrophic Lateral Sclerosis

Jeffrey D. Rothstein, MD, PhD,* Guochuan Tsai, MD,† Ralph W. Kuncl, MD, PhD,* Lora Clawson, RN, BSN,* David R. Cornblath, MD,* Daniel B. Drachman, MD,* Alan Pestronk, MD,* Barbara L. Stauch, BS,† and Joseph T. Coyle, MD†

Recently, the excitatory amino acid neurotransmitter glutamate was implicated in the pathogenesis of a variety of chronic degenerative neurological diseases in humans and animals. This report describes abnormalities in excitatory amino acids in the central nervous system of 18 patients with amyotrophic lateral sclerosis (ALS). The concentration of the excitatory amino acids glutamate and aspartate in the cerebrospinal fluid were increased significantly ($p < 0.01$) by 100 to 200% in patients with ALS. Similarly, the concentrations of the excitatory neuropeptide N-acetyl-aspartyl glutamate and its metabolite, N-acetyl-aspartate, were elevated twofold to threefold in the cerebrospinal fluid from the patients. There was no relationship between amino acid concentrations and duration of disease, clinical impairment, or patient age. In the ventral horns of the cervical region of the spinal cord, the level of N-acetyl-aspartyl glutamate and N-acetyl-aspartate was decreased by 60% ($p < 0.05$) and 40% ($p < 0.05$), respectively, in 8 patients with ALS. Choline acetyltransferase activity was also diminished by 35% in the ventral horn consistent with motor neuron loss. We conclude that excitatory amino acid metabolism is altered in patients with ALS. Based on neurodegenerative disease models, these changes may play a role in motor neuron loss in ALS.

Rothstein JD, Tsai G, Kuncl RW, Clawson L, Cornblath DR, Drachman DB, Pestronk A, Stauch BL, Coyle JT. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol 1990;28:18–25

Amyotrophic lateral sclerosis (ALS) is a chronic progressive disease of selective upper and lower motor neuron degeneration, whose pathogenesis is unknown. In several other chronic neurological disorders, such as olivopontocerebellar atrophy [1, 2] and Huntington's disease [3, 4], abnormalities of glutamate metabolism have been shown to occur and are thought to play a role in the pathophysiology of the disorder. Glutamate, the primary excitatory neurotransmitter in brain, can exert specific neurotoxic effects and can induce neuronal degeneration in vivo and in vitro [5–8]. Of particular interest, excitatory neurotoxins derived from the ingestion of cycad nuts may be, in part, responsible for the motor neuron degeneration associated with the ALS-parkinsonism-dementia complex of the Chamorro population of the Mariana islands [9, 10]. Based on the models of environmental excitatory neurotoxins [9, 10] and prior studies of human brain and spinal cord [11–16], we hypothesized that the metabolism of excitatory amino acids might be altered in patients with ALS.

To test this possibility, we examined the concentrations of glutamate, aspartate, and the excitatory neuropeptide N-acetyl-aspartyl glutamate (NAAG) in the cerebrospinal fluid (CSF) and spinal cords of patients with well-characterized, classic ALS. We report significant and specific increases in the concentrations of these excitatory compounds in the central nervous system (CNS) of patients with ALS.

Materials and Methods

The study was divided into two parts: (1) analysis of CSF from living patients and (2) analysis of autopsy material from the cervical region of the spinal cord. All biochemical analyses were performed in a blinded fashion.

CSF Analysis

CSF was collected and compared in three groups of patients (Table 1): 18 patients with ALS, 18 patients with other neurological diseases (OND), and 10 patients with hepatic encephalopathy (a comparison group expected to have high CSF levels of glutamine, to control for artifactual elevation of CSF glutamate levels).

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Table 1. Patient Characteristics in CSF Studies

Group	No.	Age (mean yr ± SEM)[a]	Duration of Disease (mo)[a]	Diagnoses[b]
Amyotrophic lateral sclerosis	18	52 ± 3.6 (31–75 yr)	15 ± 2.6 (6–36)	—
Control	18	53 ± 3.8 (22–80)	—	1° Biliary cirrhosis (2) Brainstem cerebrovascular accident (1) Cerebellar ataxia (1) Cervical myelopathy (2) Chronic inflammatory demyelinating polyneuropathy (1) Heparolenticular degeneration (1) Low back pain (1) Lumbar stenosis (1) Migraine (3) Multiple myeloma (1) Neurosyphilis (1) Pseudotumor cerebri (2) Progressive supranuclear palsy (1)
Hepatic encephalopathy	10	47 ± 4.9 (33–65)	—	—

[a] Values in parentheses represent ranges.
[b] Values in parentheses represent number of patients.

With informed consent lumbar punctures were performed in the lateral decubitus position at the L3 to L4 or L4 to L5 interspace. CSF was collected for the following studies: determinations of glucose and protein levels, cell count, and amino acid analysis. In addition, an aliquot of the first 10 ml of CSF was placed on ice immediately after lumbar puncture and either assayed for amino acids or stored at −80°C. A protocol for these studies was previously approved by The Johns Hopkins Institutional Review Board.

PATIENTS WITH ALS. We collected CSF from those patients with ALS who had lumbar puncture performed in the 2-year period from 1986 to 1988. The diagnosis of ALS was based on a rigorous set of criteria designed for a therapeutic trial now under way in our institution. Evaluations included a detailed history and physical examination, and extensive hematological, biochemical, electrophysiological, and radiological testing. The diagnosis required the presence of both upper and lower motor neuron signs, clear evidence of progression, normal nerve conduction velocities and late responses, and electromyographic evidence of diffuse denervation [17, 18]. Patients diagnosed with ALS also met an extensive list of exclusionary criteria including sensory findings; unexplained bowel or bladder changes; and anatomical, metabolic, or toxic disorders that could mimic ALS, e.g., myelopathy, lead intoxication, endocrine abnormalities, hexosaminidase A deficiency, or peripheral neuropathy. After examination of the patient's record by four neurologists, the diagnosis was accepted or rejected by consensus.

DISEASE CONTROL SUBJECTS. CSF from control patients with OND was obtained at the time of diagnostic lumbar puncture. Control patients with adequate available CSF were selected to include some patients with diseases marked by neuronal death or degeneration (see Table 1).

HEPATIC ENCEPHALOPATHY. For amino acid analysis of CSF, we studied an important comparison group with hepatic encephalopathy. Since CSF levels of glutamine are greatly elevated in that disorder [19], it provides an internal control in the study to resolve concern about artifactual elevated concentrations of CSF glutamate arising by breakdown from glutamine. All patients had chronic recurrent encephalopathy graded as clinical stage 1 or 2 [20]. All had cirrhosis, proved by evaluation of biopsy specimens, due to chronic hepatic diseases that included chronic active hepatitis, Laennec's cirrhosis, primary biliary cirrhosis, or Wilson's disease. Evidence for chronic hepatocellular disease was based on the following: presence of ascites, history of esophageal varices, decreased serum concentration of albumin (< 3.0 mg/100 ml), prothrombin ratio greater than 1.2, serum bilirubin concentration of greater than 2.5 mg/100 ml. Hepatic encephalopathy was precipitated in most patients by either gastrointestinal bleeding or increased intake of dietary protein.

Analysis of Spinal Cord Sections

SUBJECTS. Sections from the cervical region of the spinal cord were obtained at autopsy from two study groups: The ALS group consisted of 8 patients with ALS diagnosed as just described. A control group was composed of 8 patients with nonneurological disease and 1 patient with Parkinson's disease (Table 2). Both the ALS and control spinal cord specimens came from The Johns Hopkins Brain Bank (except specimens from 2 control cases were kindly supplied by the National Neurological Research Bank, Los Angeles, CA). All tissue was stored up to 2 years at −80°C. Mean age and postmortem delay were similar for both groups.

POSTMORTEM TISSUE. Analysis of the specimens was performed on 1-mm micropunch samples from ventral gray matter.

HISTOLOGY. Spinal cord specimens were routinely fixed in formalin, and then sectioned and stained with hematoxylin and eosin for pathological verification of ALS. Control spinal tissue was similarly examined.

Table 2. Patient Characteristics in Spinal Cord Studies

Group	No.	Age (mean yr ± SEM)[a]	Postmortem Delay (mean hr ± SEM)[a]	Duration of Disease (mo)[a]	Diagnoses[b]
Amyotrophic lateral sclerosis	8	62 ± 7.2 (51.0–71.0)	11 ± 6.4 (3.5–18.0)	27 ± 15 (12–48)	—
Control	9	61 ± 26 (0.5–87.0)	10.6 ± 5.5 (6.0–21.5)	—	Myocardial infarction (5) Leukemia (1) Sepsis (1) Multiple myeloma (1) Parkinson's disease (1)

[a]Values in parentheses represent ranges.
[b]Values in parentheses represent number of patients.

Amino Acid and Enzyme Assays

Aliquots of spinal cord homogenates were assayed for total protein by the method of Lowry and associates [21].

AMINO ACID ANALYSIS. Amino acid analysis of sulfosalicylic acid–treated CSF samples was performed by automated ion-exchange chromatography with lithium-based buffers on a Beckman 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA). Several aliquots were frozen and assayed at several times after the original assay, to evaluate stability of the amino acids. Evaluation of ALS and control CSF samples was done in parallel in the same assay.

N-ACETYL-ASPARTYL GLUTAMATE AND N-ACETYL-ASPARTATE ANALYSIS. NAAG and N-acetyl-aspartate (NAA) were measured in CSF and spinal cords of patients with ALS and control patients by high-performance liquid chromatography (HPLC) and in the case of NAAG, confirmed by radioimmunoassay utilizing antiserum to NAAG [22, 23]. Due to limited CSF availability, NAAG and NAA were quantified in only 12 of the 18 patients with ALS.

N-ACETYLATED-α–LINKED ACIDIC DIPEPTIDASE MEASUREMENTS. Activity of the NAAG metabolizing enzyme N-acetylated-α-linked acidic dipeptidase (NAALADase) was evaluated by measuring the hydrolysis of N-acetyl-L-aspartyl-L-[^{3}H]glutamate as described by Robinson and colleagues [22].

CHOLINE ACETYLTRANSFERASE ACTIVITY. Choline acetyltransferase (ChAT) activity in the spinal cord was determined in tissue homogenates as described by Blakely and colleagues [23]. In brief, the assay measures the synthesis of ^{14}C-acetylcholine after separation from ^{14}C-acetyl-coenzyme A (CoA) by anion exchange (AG1-X8, chloride$^-$ form) liquid chromatography.

GLUTAMATE DECARBOXYLASE ACTIVITY. Glutamate decarboxylase activity was measured in lateral cervical hemisections of the spinal cord by the method of McDonnell and Greengard [24]. The assay quantified $^{14}CO_2$ released from the α-carboxy–labeled ^{14}C-glutamate.

Statistical Analysis

Statistical analysis of data was performed by analysis of variance and *t* tests, with correction for unequal variances when appropriate, using the SAS General Linear Models Procedure (SAS Institute, Cary, NC). Values are expressed as mean ± standard error of the mean (SEM).

Table 3. CSF Amino Acid Analysis[a]

Amino Acid	Amyotrophic Lateral Sclerosis	Control	% Change
Aspartic acid	8.4 ± 1.2[b]	4.2 ± 0.6	100
Threonine	44.4 ± 3.5[c]	32.8 ± 2.0	35
Serine	55.8 ± 6.1[c]	41.1 ± 2.2	36
Asparagine	5.7 ± 0.8	5.5 ± 0.2	4
Glutamic acid	8.4 ± 1.4[b]	2.9 ± 0.4	190
Glutamine	512.6 ± 44.7	551.0 ± 19.0	−7
Glycine	26.6 ± 3.7	19.3 ± 2.7	38
Alanine	50.2 ± 4.9	38.8 ± 2.8	29
Valine	21.7 ± 1.5	18.2 ± 1.3	19
Cystine	1.7 ± 0.1	1.8 ± 0.1	6
Methionine	2.7 ± 0.4	2.7 ± 0.2	0
Isoleucine	6.9 ± 0.6	6.1 ± 0.4	13
Leucine	14.9 ± 0.9	13.7 ± 0.9	9
Tyrosine	12.5 ± 1.0	11.6 ± 1.0	8
Phenylalanine	11.6 ± 1.8	10.7 ± 0.8	8
Lysine	33.2 ± 2.7[c]	25.9 ± 1.5	28
Histidine	18.1 ± 2.0	17.8 ± 1.6	2
Arginine	25.7 ± 3.0	19.9 ± 1.0	29

[a]Values are μmol/liter (mean ± SEM).
[b]$p < 0.01$ versus control values.
[c]$p < 0.05$ versus control values.

Results

Histopathological analysis confirmed the diagnosis of ALS in all autopsy specimens. There was a characteristic loss of large motor neurons in the ventral horns of the gray matter in the cervical sections. All control specimens demonstrated intact, normal-appearing, large motor neurons in the ventral gray matter.

CSF Amino Acids, NAAG, and NAA

CSF amino acid levels in the control subjects in this experiment were not significantly different from previously published normal levels (Table 3) [25, 26]. There were no significant differences in either CSF glucose or protein levels or cell counts between any of the groups examined.

In patients with ALS, the most striking change was

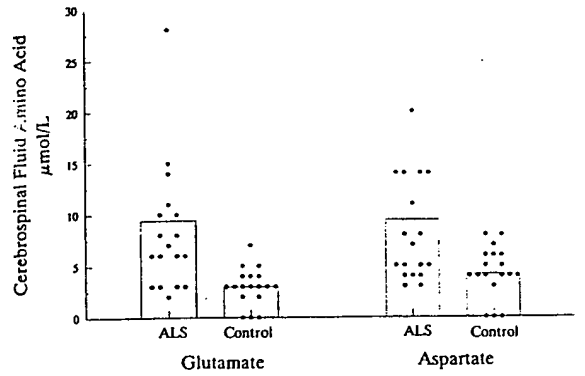

Fig 1. Concentrations of glutamate and aspartate in CSF from 18 patients with amyotrophic lateral sclerosis (ALS) compared with those found in 18 control subjects. Shaded bars represent group mean.

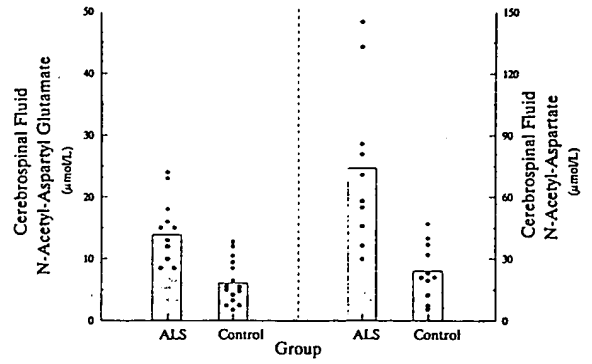

Fig 2. N-Acetyl-aspartyl glutamate and N-acetyl-aspartate concentrations in CSF from control patients and patients with amyotrophic lateral sclerosis (ALS). Shaded bars represent group mean.

the marked increase in the concentrations of the excitatory amino acids glutamate and aspartate. The average CSF concentration of glutamate was increased almost threefold ($p < 0.01$) in patients with ALS and up to tenfold in individual patients (Fig 1). Aspartate concentration was doubled ($p < 0.01$) in CSF from patients with ALS, with individual levels elevated as much as fivefold.

Concentrations of NAAG and NAA were both significantly elevated in the CSF of patients with ALS (Fig 2). NAAG concentration was more than doubled in CSF from patients with ALS ($p < 0.01$) compared to control specimens, with concentrations in individual samples increased up to fourfold. Similarly, NAA concentration was increased threefold in the patients with ALS ($p < 0.01$), again with individual levels elevated greater than fivefold compared to levels in the control CSF. NAALADase activity could not be detected in CSF specimens from either patients with ALS or control subjects.

There was no significant correlation between duration of disease and CSF aspartate, glutamate, NAAG, or NAA concentration. Furthermore, there was no observable relationship between these compounds and overall disability score or predominant pattern of dysfunction (upper or lower motor neuron signs) at the time of CSF collection.

By comparison, in 10 patients with Huntington's disease, CSF concentrations of NAAG and NAA were not different from control levels (Kurlan, Tsai, Shoulson, and Coyle, unpublished data).

There were small (< 40% above control values), statistically significant ($p < 0.05$) increases in the concentrations of the hydroxyamino acids serine and threonine in CSF from patients with ALS (see Table 3). In CSF from patients with ALS there were no significant changes in the levels of aliphatic or branched chain amino acids (alanine, glycine, valine, isoleucine, and leucine), in aromatic amino acids (tyrosine and phenylalanine), or in glutamine. Levels of basic amino acids (lysine, histidine, and arginine) were unchanged in ALS specimens, with the exception of lysine which was slightly increased by 28% ($p < 0.05$).

Amino acid concentrations are not shown for citrulline, phosphoethanolamine, hydroxyproline, α-amino-N-butyric acid, cystathione, ornithine, 1-methyl-histidine, and 3-methyl-histidine; however, they were not different between control and ALS samples of CSF.

Control Studies of Amino Acids in CSF

Glutamine is hydrolyzed to glutamate, and spontaneous hydrolysis could theoretically increase the levels of glutamate artifactually. To control for this possibility, samples of CSF from patients with hepatic encephalopathy, with marked elevations in glutamine concentrations, were also analyzed. Analysis of CSF amino acids revealed that, similar to previously published reports for chronic liver disease, glutamine was increased 2.3-fold (1,285 ± 210 μmol/liter) in patients with hepatic encephalopathy as compared to control CSF samples [19, 27]. In samples processed identically to ALS samples, the glutamate and aspartate concentrations remained normal in the CSF from patients with hepatic encephalopathy, in spite of marked elevations of glutamine. In accordance with other CSF studies, a number of CSF amino acids in patients with hepatic encephalopathy were increased; these included tyrosine, phenylalanine, methionine, threonine, and histidine (data not shown) [27].

Samples of CSF subjected to repeated analyses over a 9-month period revealed no systematic change in CSF glutamate or aspartate concentrations.

Spinal Cord Analysis

The average level of NAAG in the ventral gray horns of patients with ALS was significantly decreased by

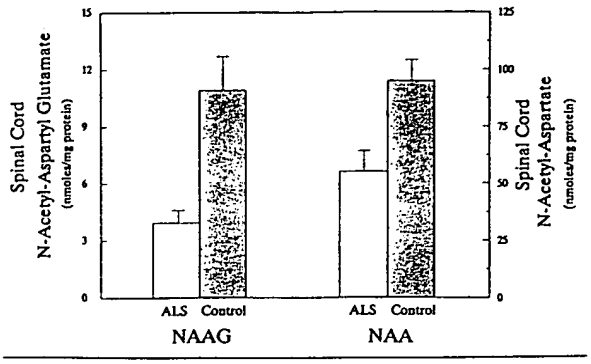

Fig 3. Concentrations of N-acetyl-aspartyl glutamate (NAAG) and N-acetyl-aspartate (NAA) in the ventral horn of patients with amyotrophic lateral sclerosis (ALS) and in control subjects. Data are presented as mean ± SEM.

Table 4. Spinal Tissue Analysis[a]

	Amyotrophic Lateral Sclerosis (n = 8)	Control (n = 9)
Choline acetyltransferase	971 ± 98[b]	1,522 ± 194
Glutamate amino decarboxylase	695 ± 92	513 ± 164
N-acetylated-α-linked acidic dipeptidase	0.148 ± 0.021	0.167 ± 0.035

[a] Values are pmol/mg of protein/hr (mean ± SEM).
[b] $p < 0.05$ compared to control values.

60% ($p < 0.02$), compared to levels in the same anatomical regions in the control cords (Fig 3). Likewise, NAA levels in ventral horn tissue of patients with ALS were decreased by 40% ($p < 0.05$). Control levels of NAAG and NAA were similar to those previously published [15]. There was no statistical relationship between NAAG levels and either disease duration or patient age.

Spinal cord NAALADase activity, present primarily in synaptic membranes, was similar in both ALS and control tissue (Table 4). Similarly, the activity of the enzyme glutamate decarboxylase, a general marker for intrinsic spinal gamma-aminobutyric acid (GABA) neurons, was unchanged in ALS spinal tissue compared to control tissue (see Table 4). By contrast, activity of the enzyme ChAT, which is present primarily in the large spinal α-motor neurons, was decreased 36% in ventral gray matter tissue compared to similar regions in control spinal cord, as expected. There was no significant correlation between the loss of ChAT activity and the spinal cord concentrations of NAAG or NAA.

Discussion

Our study showed that concentrations of the excitatory amino acids aspartate and glutamate and the neuropeptide NAAG are selectively elevated twofold to threefold in the CSF of patients with ALS. This is consistent with findings from a number of other studies that suggested alterations in the metabolism of the excitatory amino acids aspartate and glutamate in ALS [11, 12]. In postmortem studies, tissue levels of aspartate, glutamate, and GABA were reported to be diminished in several brain and spinal cord regions in ALS [12, 14, 16, 28]. In addition, studies of leukocytes and plasma have suggested that glutamate metabolism is altered in patients with ALS [29, 30]. However, study of CSF affords the advantages of allowing in vivo measurements of these amino acids within the CNS and permits studies in the early development of disease rather than at the end stage, as in postmortem analyses. Two surveys of CSF amino acids performed 12 and 20 years ago on patients with a wide variety of metabolic and degenerative diseases demonstrated somewhat elevated glutamate levels in the CSF of a small number of patients with motor neuron disease [26, 31]. Our study confirms this early finding in ALS, and extends it to the excitatory amino acid aspartate and to NAAG.

NAAG is a neuropeptide found in high concentrations in the CNS, in an uneven distribution with highest levels in the spinal cord and caudal region of the brainstem [32–34]. Immunohistochemical analysis demonstrated colocalization of NAAG to glutamatergic neurons in motor cortex, olfactory mitral cells, primary sensory afferent neurons, and lateral vestibular nuclei [35–37]. In addition, it also localizes to ChAT-positive motor neurons of both spinal and cranial nuclei, as well as some noradrenergic and adrenergic nuclei in the pons and medulla, and some serotonergic raphe nuclei [32, 38]. NAAG has been shown to be released from neural tissue by calcium-dependent processes or by electrical depolarization [39–41]. It exhibits relatively weak postsynaptic effects [42]. Following synaptic release, NAAG is metabolized via NAALADase to glutamate and NAA [22, 34]. Thus, NAAG has the properties of a neuromodulator and may be an additional source of the neurotransmitter glutamate [34].

Increased CSF levels of NAAG, NAA, and glutamate suggest the possibility of increased release and metabolism of these compounds in patients with ALS. One interpretation would be that increased release of NAAG occurs in ALS, with subsequent metabolism to glutamate and NAA leading to increased CSF concentrations of these compounds. Since the activity of NAALADase was normal in spinal cord sections from patients with ALS, the possibility that this was due to accelerated NAAG catabolism is less attractive. The low levels of NAAG in the ventral horn of the cord,

consistent with a finding described in an abstract [15], may be due to at least two factors: increased release of NAAG, perhaps by descending motor tracts, with resultant tissue depletion; or loss of α-motor neurons containing NAAG; or both. Whether these changes represent primary abnormalities in glutamate or NAAG metabolism or are a consequence of motor neuron death is not yet certain. In that regard, ChAT levels were decreased in the ventral horn of the spinal cord, as demonstrated by others [43, 44], which reflects the loss of large cholinergic motor neurons as seen on histopathological examination. Though levels of both NAAG and ChAT were reduced in the ventral horn, their lack of intercorrelation does not support the idea that the alteration in NAAG metabolism in CNS is merely secondary to motor neuron loss. The normal levels of GAD reflect intact GABA neurons in ALS spinal tissue.

The increased concentration of excitatory amino acids that we found in CSF from patients with ALS may be physiologically meaningful. In acute cerebral ischemia, extracellular glutamate concentrations increase to levels comparable to those we observed in the CSF from our patients with ALS [45]. Furthermore, the increased CSF concentration of glutamate to the 10 μmol range could be neurotoxic. There have been a large number of studies demonstrating acute glutamate toxicity in cultured neurons. Utilizing primary cerebellar granule cells, Favaron and colleagues observed 50% cell death with glutamate concentrations as low as 10 μmol.

Abnormal CSF concentrations of glutamate and aspartate may suggest altered neurotransmitter metabolism; however, only studies that are specifically directed at the neurotransmitter compartment can address these questions. The relationship between changes of CSF levels of glutamate, aspartate, and NAAG and excitatory neurotransmitter function is complex for two main reasons: (1) Glutamate metabolism in the CNS occurs in a two-compartment system and (2) CSF concentration of a compound reflects multiple processes, including altered release, uptake, cell death, or altered CSF resorption kinetics [47, 48]. Experimental studies of brain glutamate suggest that metabolism occurs in two metabolic compartments: a large compartment that is primarily for general cellular metabolism and a small compartment (10% of total) that is for neurotransmitter metabolism and is localized to the presynaptic endings and astrocytes [49–51]. Thus, measurement of total glutamate or aspartate concentration in brain tissue does not necessarily reflect the metabolism of the neurotransmitter component. At present we do not have the tools to evaluate the neurotransmitter compartment directly. Other approaches include assessing postsynaptic receptor function, and such studies are currently under way.

Previous studies demonstrated slight increases in several CSF amino acids in a number of chronic neurological diseases, e.g., Parkinson's disease [52–54]. However, the large changes in CSF concentrations of NAAG, glutamate, and aspartate reported here appear to be unique. These changes were not seen in our other patients with chronic degenerative diseases such as Huntington's disease and progressive supranuclear palsy, with acute cell damage during brainstem stroke, or with chronic spinal injury as in cervical myelopathy, and thus would suggest disease specificity to our observations.

The elevated CSF concentrations of serine, threonine, and lysine reached statistical significance in this and previous studies on motor neuron disease, but the degree of elevation was slight [11, 25, 31]. Although CSF concentrations of some amino acids may increase with age [52, 55], there was no significant difference in age between groups in our experiments.

If excitotoxicity contributes to the pathogenesis of motor neuron damage in ALS, then therapies designed to interfere with glutamate neurotransmission may be useful. To that end, Plaitakis and associates recently demonstrated that in a small group of patients with ALS, oral treatment with branched chain amino acids (valine, leucine, and isoleucine) was associated with slowing in the loss of strength [56]. The rationale for this treatment protocol was based on the potential neurotoxicity of the neurotransmitter glutamate and the assumption that administration of branched chain amino acids might alter neurotransmitter glutamate metabolism. Whether the administration of branched chain amino acids actually alters the metabolism of glutamate, aspartate, or NAAG remains to be investigated.

Excitatory amino acids and excitatory neuropeptides may be quite important in the pathogenesis of other degenerative neurological diseases. Canavan's disease, a chronic degenerative neurological disorder, was recently linked to deficiency in the enzyme that degrades NAA, aspartoacylase [57]. Two exogenous toxins that interact with glutamate receptors can also produce neuronal damage. Chronic exposure to β-N-methylamino-L-alanine, an amino acid derivative of *Cycas circinalis*, which was shown to be toxic to neurons in vitro [5, 8], appears also to cause a degenerative neurological syndrome that has some of the clinical and neuropathological characteristics of ALS [9, 10]. Furthermore, lathyrism, an upper motor neuron disorder seen after excessive ingestion of the chickling pea *(Lathyrus sativus)*, may be caused by β-N-oxalylamino-L-alanine, a potent neurotoxic stereospecific glutamate analog [9].

By analogy, the pathophysiology of ALS may be based, in part, on the abnormal chronic exposure of neurons to excitotoxic substances, such as glutamate, glutamate analogs, or excitatory neuropeptides. Increased synaptic levels of glutamate acting directly, or

acting in the absence of other "protective" mechanisms, could be responsible for neuronal death in ALS. This study demonstrates that high levels of excitatory amino acids are indeed present in the CSF of patients with ALS. These substances may, in part, be responsible for the pathogenesis of motor neuron damage. The selective loss of motor neurons in the presence of these potential excitotoxins, however, remains a puzzling question.

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European Journal of Pharmacology, 219 (1992) 173–174

Exhibit G
(10/644,645)

EJP 0365R

Rapid communication

Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity

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Intraocular pretreatment with the specific metabotropic glutamate receptor agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) in the adult rat reduced the excitotoxic effects induced in the retina by a single intraocular injection of N-methyl-D-aspartate (NMDA). Damage was estimated by assessing NMDA-induced loss of retinal choline acetyltransferase (ChAT) activity. The interaction between metabotropic and ionotropic glutamate receptors may, therefore, be considered an important target for in vivo pharmacological neuroprotection.

1S,3R-ACPD ((1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid); Metabotropic glutamate receptors; Excitotoxicity

The selective agonist for the family of metabotropic glutamate receptors (Tanabe et al., 1992), 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), besides lacking any intrinsic neurotoxicity (Koh et al., 1991a), attenuated N-methyl-D-aspartate (NMDA)-induced neuronal damage (Koh et al., 1991b) in cultured neocortical neurons. We now investigated whether stimulation of metabotropic glutamate receptors with 1S,3R-ACPD could protect the adult rat retina against NMDA-induced damage. This in vivo model of excitotoxicity was chosen because the cholinergic amacrine cells of the retina are highly sensitive to NMDA (Siliprandi et al., 1992), and the metabotropic glutamate receptor mRNAs are also very likely expressed in the amacrine cells (Tanabe-Ohuchi et al., 1992).

Male Sprague-Dawley rats (FRAR, San Pietro al Natisone, Udine, Italy; 200–250 g) were anesthetized with Equithesin and received a single 5 μl intravitreal injection of 1S,3R-ACPD (0.5–100 mM, corresponding to 2.5–500 nmoles) and vehicle (sterile phosphate buffer 0.1 M, pH 7.4) in the left and right eye, respectively. After 30 min both eyes received a 5 μl injection of 2.4 mM (12 nmol) NMDA. Retinal choline acetyltransferase (ChAT) activity was assessed 8 days later as previously reported (Siliprandi et al., 1992).

NMDA decreased the retinal ChAT activity by approximately 60% (fig. 1). Pretreatment with 1S,3R-ACPD attenuated dose dependently the neurotoxic effect of NMDA (fig. 1). 1S,3R-ACPD alone, did not produce any loss of ChAT activity or yield any histolog-

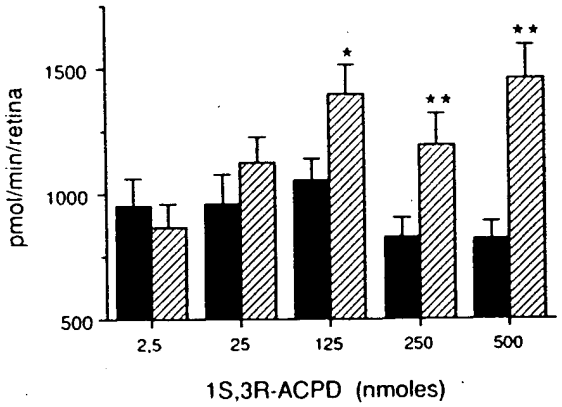

Fig. 1. Dose-response protection against NMDA-induced decrease of retinal ChAT activity by the selective metabotropic agonist 1S,3R-ACPD. Rats received an intraocular injection of vehicle (black bars) and 1S,3R-ACPD (stripped bars) in the right and left eye, respectively, 30 min prior to the intraocular injection of 12 nmol of NMDA. Each value represents the mean ± S.E.M. (n = 13). * $P < 0.05$; ** $P < 0.001$, Student's t-test. The data were derived from four independent experiments in which the values of retinal ChAT activity ranged from 2059.6 ± 19.2 to 2216.5 ± 41.32 pmol/min per retina in control, non-injected retinae and from 2123.9 ± 30.65 to 2278.9 ± 24.87 pmol/min per retina in retinae injected with vehicle. Intraocular administration of 1S,3R-ACPD alone, at all doses tested, did not alter retinal ChAT activity (i.e. 2164.8 + 63.3 pmol/min per retina after administration of 500 nmol of 1S,3R-ACPD).

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ical evidence of retinal alterations (data not shown). Hence, the in vivo stimulation of metabotropic glutamate receptors could reduce NMDA-induced neurotoxicity, thus confirming the results of Koh et al. (1991b) obtained in vitro. However, according to the latter results, the protective effect of 1S,3R-ACPD was transient, i.e. changed during the culture period. The reason for this could be, in part, the exceptional plasticity of metabotropic glutamate receptor expression in primary neuronal cultures (Favaron et al., 1992).

Our results might indicate that 1S,3R-ACPD modulates the NMDA receptors present on the cholinergic amacrine cells in the retina via intracellular events triggered by activation of this/those metabotropic glutamate receptors expressed by the same cells. It should be mentioned, however, that another type of NMDA-metabotropic glutamate receptor interaction also exists in which stimulation of metabotropic glutamate receptors by 1S,3R-ACPD potentiates the NMDA-mediated toxicity (McDonald and Schoepp, 1992). Such a discrepancy could be due to a differential expression of the various metabotropic glutamate receptors (Tanabe et al., 1992) whose functional significance is still unclear. Whether the specific pharmacology of metabotropic glutamate receptors is a promising way to limit glutamate excitotoxicity has to be explored. If so, use of agonists different from 1S,3R-ACPD and selective for a particular type of metabotropic glutamate receptor might become a novel strategy for treating brain pathologies such as stroke, trauma or chronic neurodegeneration.

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ELSEVIER

Exhibit H (10/644,645)

European Journal of Pharmacology 256 (1994) 109–112

ejp

Short communication

Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death

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Abstract

(2*S*,1'*R*,2'*R*,3'*R*)-2-(2,3-Dicarboxycyclopropyl)glycine (DCG-IV), a potent agonist of subtypes 2 and 3 of metabotropic glutamate receptors (mGluR2 or 3), protected cultured cortical neurons against excitotoxicity induced either by a brief exposure to *N*-methyl-D-aspartate (NMDA) or a prolonged exposure to kainate. As a neuroprotective agent, DCG-IV was much more potent than the mixed agonists 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) or (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I), suggesting a neuroprotective role for mGluR2 or 3 against excitotoxic neuronal death.

Key words: DCG-IV ((2*S*,1'*R*,2'*R*,3'*R*)-2-(2,3-dicarboxycyclopropyl)glycine); Excitotoxicity; Metabotropic glutamate receptor; Cortical neuron

1. Introduction

Although metabotropic glutamate receptors (mGluRs) have been extensively characterized, their role in neuronal toxicity is still a matter of debate. Studies with 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) – a mixed agonist at all mGluR subtypes – have indicated that activation of mGluRs may either facilitate or prevent neuronal degeneration depending on the cell type (or brain region) examined, as well as on the experimental measures of neurotoxicity (for a review, see Schoepp and Conn, 1993). It is therefore likely that distinct mGluR subtypes differentially influence neuronal toxicity and that the final result will reflect the proportion of neurotoxic and neuroprotective mechanisms elicited by mGluRs within a discrete brain region. (2*S*,1'*R*,2'*R*,3'*R*)-2-(2,3-Dicarboxycyclopropyl)glycine (DCG-IV) has been recently introduced as a potent mGluR ligand capable of depressing monosynaptic transmission in the spinal cord (Ishida et al., 1993; Ohfune et al., 1993). In brain slices, DCG-IV inhibits forskolin-stimulated cAMP formation without affecting basal polyphosphoinositide hydrolysis (Genazzani et al., 1993). Studies in transfected cells have shown that DCG-IV potently activates the mGluR2 and 3 receptor subtypes, with very low activity on mGluR4 and no activity on mGluR1. In cells transfected with mGluR2 or 3, DCG-IV is considerably more potent than L-glutamate to inhibit forskolin-stimulated cAMP formation (Hayashi et al., 1993). We now report that DCG-IV protects cultured cortical neurons against the excitotoxic activity of kainate or *N*-methyl-D-aspartate (NMDA), with a much greater potency than the mixed mGluR agonists 1*S*,3*R*-ACPD or (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl) glycine (L-CCG-I).

2. Materials and methods

2.1. Materials

NMDA, kainic acid and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO,

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USA) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]-cyclohepten-5,10-imine hydrogen maleate (MK-801) from RBI (Natick, MA, USA). 1*S*,3*R*-ACPD was obtained from Tocris Neuramin (Essex, UK). DCG-IV was provided by Dr. H. Shinozaki. L-CCG-I was provided by Prof. R. Pellicciari (University of Perugia, Italy).

2.2. Cell cultures

Excitotoxic neuronal death was studied in murine cortical primary cultures containing both neurons and astrocytes. Cultures were prepared as described by Choi et al. (1987) and used at 14–16 days in vitro. Cultures of pure astrocytes were prepared as described by Rose et al. (1992) and used as controls.

2.3. Estimation of slow and fast excitotoxicity

NMDA toxicity was induced by incubating the cultures for 10 min ('fast toxicity') at room temperature in a Hepes-buffered balanced salt solution containing submaximal concentrations of NMDA (100 μM) in the absence or presence of mGluR agonists co-added with the drug. The cultures were then returned to culture medium lacking serum and glutamine (stock medium) and toxicity was evaluated 24 h later. Kainate toxicity was induced by incubating cultures for 24 h ('slow toxicity') with submaximal concentrations of kainate (60 μM) in the absence or presence of mGluR agonists (added at the same time as kainate) in stock medium containing 10 μM MK-801 to prevent any secondary activation of NMDA receptors. Overall neuronal cell injury was estimated by phase-contrast microscopy, trypan blue exclusion or by measuring lactate dehydrogenase (LDH) activity released by damaged neurons in the incubation medium.

2.4. Measurement of polyphosphoinositide hydrolysis

Stimulation of polyphosphoinositide hydrolysis was measured as follows: mixed or pure glial cultures were incubated for 18 h with 1 μCi of *myo*-[2-^{3}H]inositol (NEN-DuPont, spec. act. 15.6 Ci/mmol) for the labelling of inositolphospholipids. The cultures were then shifted into stock medium containing 10 μM MK-801 and were stimulated with mGluR ligands in the presence of 10 mM Li^+ for 30 min. [^{3}H]Inositolmonophosphate (InsP) formation was measured as described previously (Nicoletti et al., 1986).

2.5. Measurement of cAMP formation

Mixed or glial cortical cultures were shifted into stock medium containing the phosphodiesterase inhibitor, IBMX (500 μM), and incubated for 10 min in the presence of forskolin and/or mGluR ligands. The incubation was terminated by addition of 0.4 N $HClO_4$ after complete removal of the medium. The cells were scraped and the suspension was added to K_2CO_3 (0.4

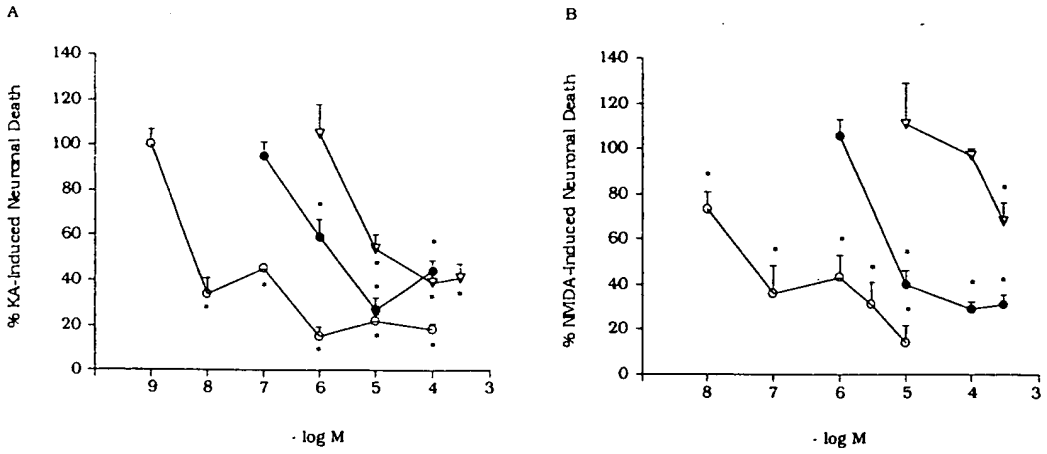

Fig. 1. Neuroprotective effect of DCG-IV (open circle), 1*S*,3*R*-ACPD (triangle) or L-CCG-I (filled circle) in primary cultures of cortical cells exposed to kainate (A) or NMDA (B). The data represent the number of cells stained with trypan blue and are expressed as percent of kainate- or NMDA-induced neuronal degeneration. Identical results were obtained by measuring LDH activity released from damaged neurons in the incubation medium. Each data point is the mean of 6–24 determinations from at least two individual experiments. In a representative 'slow toxicity' experiment, the number of cells stained with trypan blue in three microscopic fields was: 9.7 ± 0.8 (basal) or 143 ± 8.2 (24 h exposure to kainate); in a representative 'fast-toxicity' experiment, the number of cells stained with trypan blue in three microscopic fields was: 19 ± 5.3 (basal) or 271 ± 38 (10 min exposure to NMDA). * $P < 0.05$ (one-way analysis of variance (ANOVA) and Fisher PLSD test), as compared to the values obtained in the absence of mGluR agonists.

Table 1
DCG-IV fails to stimulate polyphosphoinositide hydrolysis in primary cultures of cortical cells

	[^{3}H]InsP formation (d.p.m./well)
Basal	5900 ± 100
L-CCG-I (100 μM)	11000 ± 1000 [a]
1*S*,3*R*-ACPD (100 μM)	12000 ± 1800 [a]
DCG-IV (10 μM)	5300 ± 880

The values are the means ± S.E.M. of six individual determinations. Incubations were performed in the presence of 10 μM MK-801.
[a] $p < 0.05$ vs. basal or DCG-IV (ANOVA and Fisher PLSD test).

N final) and centrifuged at low speed. cAMP concentration in the supernatant was quantitated with a commercially available kit (NEN Dupont, NEC 033).

3. Results

Brief exposure to NMDA or continuous exposure to kainate led to a substantial increase (at least 10-fold) in the number of neurons stained with trypan blue, as well as in the LDH activity released in the medium (5–15-fold). MK-801 (10 μM) totally prevented the neurotoxic effect of NMDA but did not influence the action of kainate (not shown). NMDA or kainate did not affect cell viability in cultures of pure astrocytes (not shown). Addition of DCG-IV during the time of exposure to NMDA or kainate dramatically reduced the extent of neuronal degeneration. The neuroprotective effect of DCG-IV was concentration-dependent, with apparent IC_{50} values of 5 and 30 nM against kainate- or NMDA-induced toxicity, respectively (Fig. 1A and B). L-CCG-I and 1*S*,3*R*-ACPD were in general more potent against kainate- than against NMDA-induced toxicity; however, both drugs were much less potent than DCG-IV on both measures of neuronal degeneration (Fig. 1A and B).

Both 1*S*,3*R*-ACPD and L-CCG-I (100 μM) increased [^{3}H]InsP formation in cultured cortical cells, whereas DCG-IV (10 μM) was inactive (Table 1). None of these drugs induced changes in [^{3}H]InsP formation in pure glial cultures (not shown). In both mixed and glial cultures, forskolin stimulated cAMP formation to about the same extent; cAMP levels were: basal, 3.8 ± 0.4 and 3.3 ± 1.3; forskolin (10 μM), 23 ± 2.8 and 30 ± 4.7 pmol/well in mixed and glial cultures respectively. Forskolin-stimulated cAMP formation was not affected by any of the mGluR agonists in any of these cultures (not shown).

4. Discussion

mGluRs form a family of at least seven subtypes (named mGluR1–7) grouped into three major classes. mGluR1 and 5, which are potently activated by quisqualate, are coupled to polyphosphoinositide hydrolysis; mGluR2 and 3 are negatively linked to adenylyl cyclase activity and preferentially activated by L-CCG-I and 1*S*,3*R*-ACPD; mGluR4, 6 and 7 are also negatively linked to adenylyl cyclase activity, but are activated by L-2-amino-4-phosphonobutanoate (Nakanishi, 1992; Sangstad et al., 1993). The action of L-CCG-I and 1*S*,3*R*-ACPD recruits both mGluRs coupled to PPI hydrolysis and those linked to adenylyl cyclase (reviewed in Nakanishi, 1992). These drugs therefore cannot be used to determine the relative contribution of different subtypes to the pathophysiology of excitotoxic neuronal degeneration. DCG-IV has recently been described as a potent mGluR2 or 3 receptor agonist (Hayashi et al., 1993), although it also activates NMDA receptors with a lower potency (Ishida et al., 1993). Whereas we have confirmed the lack of activity of DCG-IV on polyphosphoinositide hydrolysis, inhibition of forskolin-stimulated cAMP formation could not be detected in our cultures, as astrocytes, which did not respond to mGluR agonists, made a major contribution to the final effect of forskolin on cAMP formation. DCG-IV was highly potent in protecting cultured cortical neurons against both the fast toxicity induced by NMDA and the slow toxicity induced by kainate, two models that have in common a late development of necrotic neuronal death. This suggests that activation of mGluR2 or 3 receptors is neuroprotective in cultured cortical cells. Accordingly, L-CCG-I is more potent than 1*S*,3*R*-ACPD both to protect cortical neurons (present data) and activate mGluR2 receptors in transfected cells (Nakanishi, 1992). Whether the neuroprotective activity of these drugs is related to the inhibition of adenylyl cyclase or to other mechanisms triggered by mGluR activation, such as inhibition of voltage-sensitive Ca^{2+} channels or modulation of K^+ channels (see Schoepp and Conn, 1993 for a review) remains to be established. Whatever the mechanism, DCG-IV was the most potent neuroprotective agent in the present study, suggesting that the drug should be tested in experimental models of acute and chronic neurodegenerative diseases.

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Exhibit I (10/644,645)

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ejp

Activation of group III metabotropic glutamate receptors is neuroprotective in cortical cultures

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Abstract

(*RS*)-α-Methyl-4-phosphonophenylglycine (MPPG) and (*S*)-α-methyl-3-carboxyphenylalanine (M3CPA), two novel preferential antagonists of group III metabotropic glutamate (mGlu) receptors, antagonized the neuroprotective activity of L-2-amino-4-phosphonobutanoate (L-AP4) or L-serine-*O*-phosphate in mice cultured cortical cells exposed to a toxic pulse of *N*-methyl-D-aspartate. In contrast, MPPG did not influence the neuroprotective activity of the selective group II mGlu receptors agonist, (2$_S$,1'$_R$,2'$_R$,3'$_R$)-2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV). These results indicate that activation of group III mGlu receptors exerts neuroprotective activity against excitotoxic neuronal death. At least one of the two major group III mGlu receptor subtypes, i.e. mGlu$_4$ receptor, is expressed by cultured cortical neurons, as shown by immunocytochemical analysis with specific polyclonal antibodies.

Keywords: Metabotropic glutamate receptor; Excitotoxicity; Cortical neuron

1. Introduction

Metabotropic glutamate (mGlu) receptors form a family of at least 8 subtypes, which have been classified into three groups, based on sequence homology, pharmacological profile of activation and transduction pathways (reviewed in Nakanishi, 1994; Pin and Duvoisin, 1995). Group I includes mGlu$_1$ and mGlu$_5$ receptors, which are coupled to polyphosphoinositide (PI) hydrolysis in transfected cells and are preferentially activated by quisqualate and (*S*)-3,5-dihydroxyphenylglycine (DHPG). Group II includes mGlu$_2$ and mGlu$_3$ receptors, which are negatively linked to adenylyl cyclase and are potently activated by (2$_S$,1'$_R$,2'$_R$,3'$_R$)-2-dicarboxycyxlopropyl) glycine (DCG-IV). Members of group III (mGlu$_{4,6\text{-}8}$ receptors) are also negatively linked to adenylyl cyclase in transfected cells and are selectively activated by L-2-amino-4-phosphonobutanoate (L-AP4) or by its analogue, L-serine-*O*-phosphate. The neuroprotective activity of group II mGlu receptor agonists has been recently documented (Bruno et al., 1994, 1995; Buisson and Choi, 1995; Buisson et al., 1996). L-AP4 and L-serine-*O*-phosphate can also partially protect cultured neurons against degeneration induced by excitotoxins (Bruno et al., 1995), β-amyloid peptide (Copani et al., 1995) or nitric oxide (Maiese et al., 1995). Although these effects have been related to the activation of group III mGlu receptors, conclusive evidence is lacking. L-AP4 and L-serine-*O*-phosphate exert multiple actions in brain tissue. They are, for example, good substrates for the Ca^{2+}/Cl^--dependent glutamate transporter (Monaghan et al., 1983; Fagg and Foster, 1983), and inhibit mGlu receptor agonist-stimulated PI hydrolysis in a non-competitive fashion (Nicoletti et al., 1986; Schoepp and Johnson, 1988). We now report that two novel group III mGlu receptor antagonists selectively prevent the neuroprotective action of L-AP4 and L-serine-*O*-phosphate in cultured cortical neurons, and that these neurons express the mGlu$_4$ receptor protein. This supports the view that group III mGlu receptors behave as neuroprotective receptors in the CNS.

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2. Materials and methods

2.1. Characterization of $mGlu_4$ receptor antibodies

Polyclonal antibodies were raised in rabbits against a synthetic peptide corresponding to the following non-conserved carboxy-terminal amino-acid sequences (one-letter code): CLETPALATKQTYVTYTNHAI for $mGlu_4$ receptor and PAKKKYVSYNNLVI for $mGlu_7$ receptor. Antibodies were purified by immunoaffinity chromatography with peptide-coupled Affigel 10/15 (Bio-Rad). The specificity of the antibody was tested in human embryonic kidney (HEK) 293 cells transfected with either $mGlu_4$ and $mGlu_7$ receptors, as follows. HEK 294 cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum were transfected with 4 μg of plasmid DNA per dish (density = 5×10^{-5} cells) by calcium phosphate precipitation. Cell monolayers were harvested 48 h after transfection by a 10-min incubation on ice with 300 μl of lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl^2$, 1 mM EGTA; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulphonyl fluoride; 0.5 μg/ml leupeptin; 1 μg/ml pepstatin; 1 μg/ml aprotinin). Twenty μg of protein from cell lysates was separated by electrophoresis on 8% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes by standard procedures in 0.2 M phosphate buffer. The blots were blocked for 1 h with NETG buffer (150 mM NaCl; 5 mM EGTA; 50 mM Tris-HCl pH 7.4; 0.05% Triton X-100; 0.25% gelatine) and incubated for 1 h at room temperature with the primary and secondary antibodies. The primary antibodies were diluted 1 : 1000 in NETG buffer, the secondary antibody (peroxidase-coupled goat anti-rabbit, Bio-Rad) was diluated 1 : 1500. After each antibody incubation of 1 h, the blots were washed in NETG for 1 h. Detection was performed using the enhanced chemioluminescence (ECL) Western blotting analysis system (Amersham).

2.2. Preparation of cortical cell cultures

Mixed cortical cultures containing both neurons and glia were prepared from fetal mice at 14–16 days of gestation, as described previously (Rose et al., 1993). Briefly, dissociated cortical cells were plated in 15-mm multiwell vessels (Nunc) on a layer of confluent glial cells (7–14 days in vitro), using a plating medium of Eagle's minimal essential medium (MEM-Earle's salts, supplied glutamine free) supplemented with 5% heat-inactivated horse serum, 5% fetal calf serum, glutamine (2 mM) and glucose (final concentration 21 mM). Cultures were kept at 37°C in a humified 5% CO_2 atmosphere. After 3–5 days in vitro, non-neuronal cell division was halted by 3-day exposure to 10 μM cytosine arabinoside, and cultures were shifted to a maintenance medium identical to the plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice per week. Only mature cultures (13–14 days in vitro) were used for the experiments.

2.3. Immunocytochemistry

Cortical cultures at 13–14 days in vitro were stained with $mGlu_4$ receptor antibodies. Cells were washed twice with phosphate-bufferd saline (PBS), fixed for 30 min 2% paraformaldehyde, washed three times with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then washed, blocked with serum and incubated with the primary antibody (1 : 1000) for 2 h at room temperature. After cells were washed three times, the secondary antibody (1 : 200) was added for 1 h. After the reaction with avidin-biotin-horseradish peroxidase (Vectastain ABC-Elite kit; Vector Labs, Burlingame, CA, USA), staining was developed by exposure to 0.5% diaminobenzidine/0.01% H_2O_2 (2–10 min).

2.4. Exposure to excitatory amino acids

Exposure to *N*-methyl-D-aspartate (NMDA) (10 min), in the presence or absence of mGlu receptor agonists and antagonists, was carried out in mixed cortical cultures at room temperature in a HEPES-buffered salt solution (HBSS) containing (in mM): NaCl, 120: KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 20; glucose, 15. After 10 min, the drugs were washed out and the cultures were incubated at 37°C for the following 24 h in medium stock (Eagle's minimal essential medium, supplemented with 15.8 mM $NaHCO_3$ and glucose up to 25 mM), and then assessed for neuronal injury.

2.5. Assessment of neuronal injury

Neuronal injury was estimated by examination of the cultures with phase-contrast microscopy at 100 ×, 24 h after the insult. Neuronal damage was quantitatively assessed in all experiments by estimation of dead neurons by Trypan blue staining. Stained neurons were counted from three random fields per well. Neuronal injury was also assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged or destroyed cells into the extracellular medium, as described in Koh and Choi (1987).

2.6. Materials

N-methyl-D-aspartate (NMDA) and L-serine-*O*-phosphate were obtained from Sigma (St. Louis, MO, USA); L-AP4 was purchaed from Tocris Cookson (Bristol, UK); DCG-IV was kindly provided by Dr. H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Japan). (2_S,$1'_S$,$2'_S$)-2-methyl-2-(2′-carboxycyclopropyl) glycine (MCCG-I), (*RS*)-α-methyl-4-phosphonophenylglycine

(MPPG) and (*S*)-α-methyl-3-carboxyphenylalanine (M3CPA) were synthesized by Dr. D.E. Jane (University of Bristol, UK).

3. Results

3.1. Characterization of $mGlu_4$ receptor antibodies

In lysates from cells transfected with $mGlu_{4a}$ receptor cDNA, $mGlu_4$ receptor antibodies labeled a major band at about 100 kDa, which corresponds to the deduced molecular weight of the receptor, and an additional band of higher

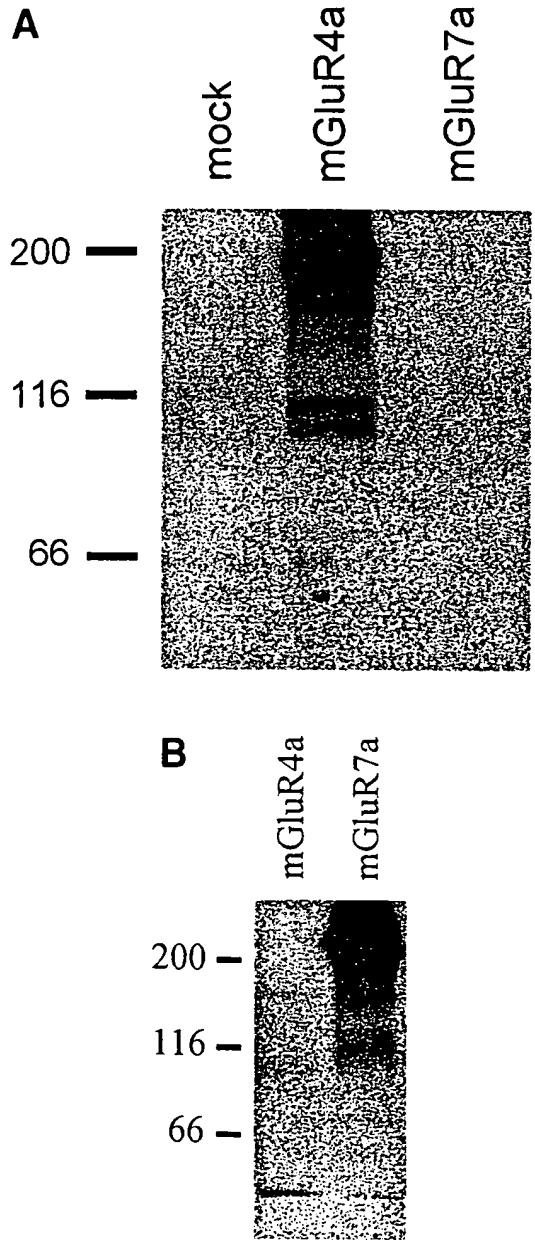

Fig. 1. Immunoblotting with purified mGluR4 (A) or mGluR7 (B) anbitodies in lysates from cells transfected with plasmids encoding mGluR4 and mGluR7 or with a control plasmid. Molecular weight markers are shown on the left side of each figure.

Table 1
Group III mGlu receptor antagonists reverse the protective effect of L-serine-*O*-phosphate against NMDA-induced toxicity

	% NMDA-induced toxicity (Trypan blue staining)		
		MPPG 30 μM	M3CPA 30 μM
NMDA 100 μM	100 ± 2	98 ± 5	95 ± 5
+ L-SOP 100 μM	54 ± 4	113 ± 12 [a]	81 ± 7 [a]

Values are the means ± S.E.M. of 8 individual determinations from two different experiments. [a] $P < 0.05$ vs. NMDA + L-SOP (ANOVA + Fisher PLSD test). L-SOP = L-serine-*O*-phosphate.

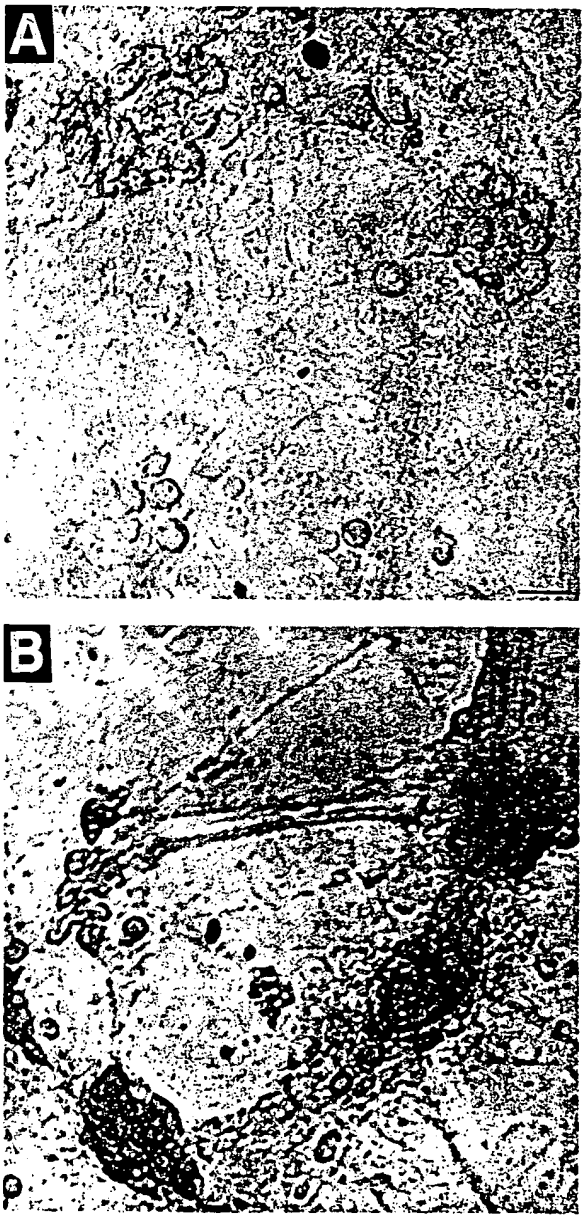

Fig. 2. Immunocytochemistry of mixed cortical cells stained with mGluR4 antibodies (B). Non-specific staining is shown for comparison in a sister culture not exposed to the mGluR4 antibody (A). Bar, 16 μm.

molecular weight, which probably represents a polymeric aggregate of the receptor. $mGlu_4$ receptor antibodies did not react with an protein in lysates from cells transfected with either $mGlu_{7a}$ receptor or a control plasmid (Fig. 1a). In contrast, lysates from cells transfected with $mGlu_{7a}$ (but not $mGlu_{4a}$) receptor cDNA were immunoreactive to $mGlu_7$ receptor antibodies (Fig. 1b).

3.2. Immunocytochemistry with $mGlu_4$ receptor antibodies in cortical neurons

Cultured cortical neurons were immunoreactive to $mGlu_4$ receptor antibodies, which appeared to stain both cell bodies and neurites (Fig. 2).

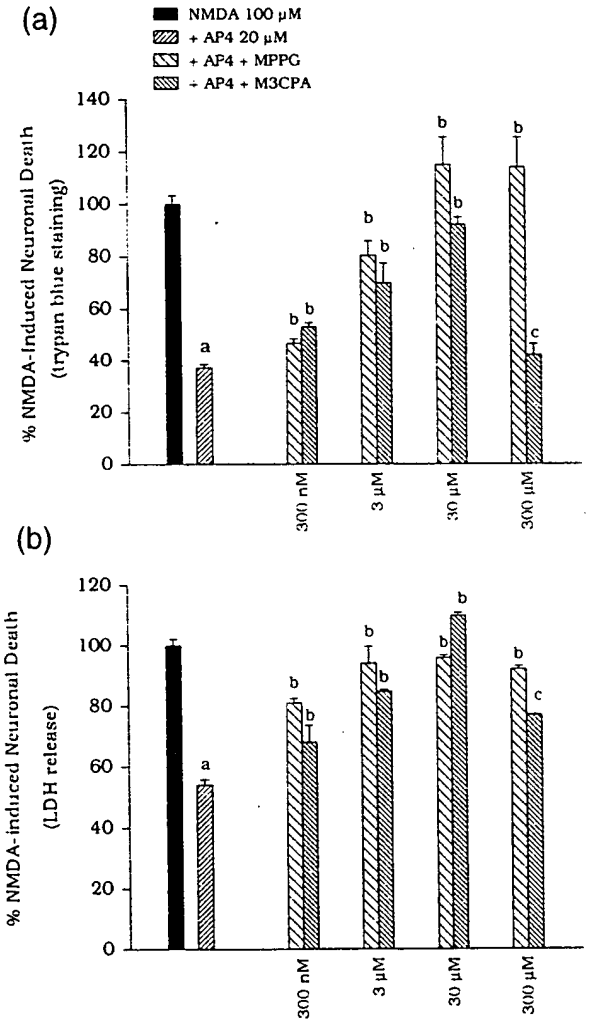

Fig. 3. MPPG or M3CPA antagonize the neuroprotective effect of L-AP4 in a concentration-dependent fashion. (A) Data refer to the number of cells stained with Trypan blue (mean ± S.E.M.) and are expressed as percentage of NMDA-induced neuronal death. Values were calculated from 8–12 individual determinations (2 or 3 independent experiments). (B) Extracellular LDH activity was measured in parallel in the same experiment. [a] $P < 0.05$ vs. NMDA; [b] $P < 0.05$ vs. NMDA + AP4; [c] $P < 0.05$ vs. NMDA + AP4 + M3CPA 30 μM (one-way ANOVA + Fisher PLSD test). Neither MPPG or M3CPA influenced per se NMDA toxicity at any concentration tested.

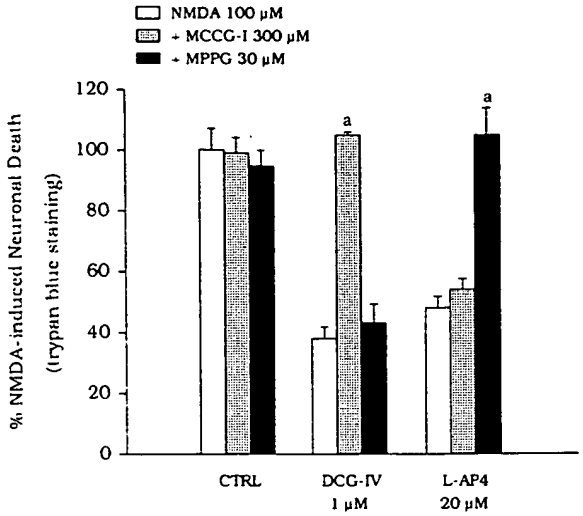

Fig. 4. Influence of MCCG-I or MPPG on the neuroprotective activity of DCG-IV or L-AP4 against NMDA toxicity. Values refer to the number of dead neurons, as stained by Trypan blue and were calculated from 8 individual determinations (2 independent experiments). [a] $P < 0.05$ (one-way ANOVA + Fisher PLSD test), if compared with the respective values obtained in the absence of antagonists.

3.3. Toxicity studies

A 10-min exposure to maximal concentrations of NMDA (> 150 μM) induced necrotic death in about 90% of cortical neurons, as indicated by both Trypan blue staining and LDH release. In our experiments, we used a submaximal concentration of NMDA (100 μM), which produced the death of 75–80% of neurons (here indicated as 100% of NMDA toxicity). As expected (Bruno et al., 1995), L-AP4 partially protected cortical neurons against NMDA toxicity in a concentration-dependent fashion, with an apparent EC_{50} value of 1 μM (not shown). Two novel group III mGlu receptor antagonists, MPPG and M3CPA, were challenged against a maximally effective concentration of L-AP4 (20 μM). Neither MPPG nor M3CPA (up to 300 μM) influenced per se neuronal viability or NMDA-induced neuronal death (not shown). Both drugs instead antagonized the neuroprotective effect of L-AP4 in a concentration-dependent fashion (IC_{50} between 1 and 3 μM for both MPPG and M3CPA) (Fig. 3a,b). At high concentrations, however, M3CPA was less effective in antagonizing neuroprotection by L-AP4 (although this tendency was more evident by using Trypan blue staining rather than LDH release for the detection of neuronal toxicity) (Fig. 3). Both MPPG and M3CPA (the latter at 30, but not 100 μM) also antagonized the neuroprotective effect of L-serine-*O*-phosphate (Table 1). Finally, we challenged the neuroprotective action of DCG-IV (1 μM) or L-AP4 (20 μM) with the selective class II mGlu receptor antagonist, MCCG-I or with MPPG. As expected, the neuroprotective activity of DCG-IV was antagonized by MCCG-I (300 μM), but not by MPPG at a concentration (30 μM) that

was maximally effective against L-AP4. In contrast, the neuroprotective action of L-AP4 was insensitive to MCCG-I (Fig. 4).

4. Discussion

L-AP4, a close structural analogue of glutamate, has long been known to depress excitatory synaptic transmission (Koerner and Cotman, 1981; Davies and Watkins, 1982; Baskys and Malenka, 1991; Rainnie and Shinnick-Gallagher, 1992) and to reduce glutamate release (Jones and Roberts, 1990; Vasquez et al., 1995). L-AP4 and its analogue L-serine-*O*-phosphate have been shown to partially protect cultured cortical neurons against excitotoxic degeneration (Bruno et al., 1995). The recent progress in the pharmacology of mGlu receptors now makes it possible to establish whether this neuroprotective effect is mediated by the activation of group III mGlu receptors. A series of compounds bearing an α-methyl group have been described as preferential antagonists of group III mGluRs. We used MPPG and M3CPA (Kemp et al., 1994; Roberts et al., 1994; Roberts, 1995) rather than α-methyl-AP4 (Jane et al., 1994), because the latter exhibits low potency and mimics the action of L-AP4 in inhibiting forskolin-stimulated cAMP formation in rat cortical slices (Kemp et al., 1994; Gottesman et al., 1995; Roberts, 1995). MPPG is an analogue of (*RS*)-α-methyl-4-carboxymethylphenylglycine (MCPG), in which the substitution of a carboxyl by a phosphonate group in position 4 lowers the affinity of the compound for group I and II mGluRs and substantially enhances the affinity for group III (Jane et al., 1995; Roberts, 1995). MPPG binds to and activates recombinant human $mGlu_4$ receptor expressing cells (M.A. Tones, P.J. Flor, R. Kuhn and T. Knoepfel, personal communication). Both MPPG and M3CPA substantially antagonized the neuroprotective effect of L-AP4 in cultured cortical neurons, and their apparent IC_{50} values fell in the same range as those reported in electrophysiological experiments (Jane et al., 1995). M3CPA, however, showed a tendency to have a bell-shaped concentration-response curve, because it appeared to be less effective at 300 than at 30 μM. The action of MPPG and M3CPA was specific for L-AP4 and L-SOP, because they did not influence the neuroprotective action of the group II mGlu receptor agonist, DCG-IV. In contrast, MCCG-I, a selective antagonist of group II mGlu receptors (Jane et al., 1994), antagonized the action of DCG-IV, but was inactive when used to challenge L-AP4. Taken together, these results indicate that the neuroprotective effect of L-AP4 and L-SOP in cultured cortical neurons was mediated by the activation of group III mGlu receptors. We have therefore studied the expression of $mGlu_4$ receptors in mixed cortical cultures. Cortical neurons were immunopositive for $mGlu_4$ receptors, and immunostaining was present on both neuronal cell bodies and neurites, as one can expect based on the putative presynaptic location of $mGlu_4$ receptors. It is conceivable that cortical cultures express also $mGlu_7$ receptors, as a high expression of $mGlu_7$ receptor mRNA is observed in neuronal cells throughout the cerebral cortex (Okamoto et al., 1994; Saugstad et al., 1994). However, a major involvement of $mGlu_4$ receptors is suggested by the high potency exibited by L-AP4 (EC_{50} = 1 μM) as a neuroprotectant, according to what is observed in transfected cells, where L-AP4 interacts with $mGlu_4$ receptors in the high nanomolar range, but interacts with $mGlu_7$ receptors at much higher concentrations (reviewed in Pin and Duvoisin, 1995).

In conclusion, the present results provide clear-cut pharmacological evidence that activation of class III mGlu receptors protects cultured cortical neurons against excitotoxic death. Class III mGlu receptors therefore become a putative target for the design of novel neuroprotective agents.

Acknowledgements

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Glutamate receptor antagonists protect against ischemia-induced retinal damage

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Abstract

The effects of intravitreal injections of excitatory amino acid receptor antagonists have been studied on the ischemic neuronal damage induced by photochemical occlusion of the retinal vessels. Rats were systemically injected with rose bengal fluorescein dye and one of their eyes was exposed to bright light. The activities of the enzymes, choline-acetyltransferase and glutamate decarboxylase, were measured as an index of neuronal loss in the lesioned tissue. Lesioned retinas had a 75 ± 5% reduction in choline-acetyltransferase activity and a 72 ± 8% reduction in glutamate-decarboxylase activity, suggesting that the lesion causes a massive loss of retinal neurons, which use acetylcholine or γ-aminobutyric acid (GABA) as neurotransmitter. A single intravitreal injection of excitatory amino acid receptor antagonists, performed immediately after the lesion, significantly reduced this loss. Both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate) (NMDA) types of ionotropic glutamate receptor antagonists were active in a dose-dependent manner. Almost complete protection was also obtained with relatively large doses of thiokynurenic acid (400 nmol), a non-selective antagonist of both AMPA and NMDA glutamate receptors, while 7-Cl-thiokynurenic acid, a potent and selective glycine receptor antagonist, was not active up to 200 nmol. These results strongly suggest that excitotoxic mechanisms are involved in ischemia-induced neuronal death in the retina and that appropriate treatments with antagonists of both AMPA and NMDA receptor types may significantly reduce this damage.

Keywords: Retina; Excitatory amino acid receptor antagonist; AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid); NMDA (*N*-methyl-D-aspartate); Thiokynurenic acid; Rose bengal

1. Introduction

Appropriate doses of excitatory amino acid receptor antagonists significantly reduce the neuronal death induced by hypoxic or ischemic insults to the brain (Rothman and Olney, 1986; Meldrum, 1985; Albers et al., 1989). When the ischemic insult is characterized by an almost complete lack of blood supply to a specific brain region (focal ischemia), competitive and non-competitive NMDA (*N*-methyl-D-aspartate)) receptor antagonists are particularly active. In fact, they are able to reduce the extent of tissue damage in animal models of ischemia in rats (Park et al., 1988), rabbits (Steinberg et al., 1988) and mice (Gotti et al., 1990). AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) antagonists prevent delayed neuronal death in models of global ischemia (Sheardown et al., 1990; Buchan et al., 1991). A recently introduced strategy for studying the pathophysiology of brain ischemia consists of the intravenous injection of the fluorescein dye, rose bengal, and the appropriate illumination of a restricted area of the brain cortex (Watson et al., 1985; Van Reemps et al., 1987; De Ryck, 1990). The illumination causes a photochemical reaction characterized by the production of oxygen derived free radicals (Kusama et al., 1989), which are able to damage the endothelia and to start the processes of platelet aggregation, thrombus formation and eventual occlusion of blood vessels supplying the illuminated area. This type of tissue damage can also be induced in the retina (Royster et al., 1988; Mosinger and Olney, 1989; Moroni et al., 1993). In this organ, glutamate receptors of NMDA and AMPA types

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are present in several neuronal populations (Miller and Slaughter, 1986; Muller et al., 1992; Hamassaki-Britto et al., 1993) and it is widely accepted that abnormal stimulation of these receptors may result in the death of retinal neurons (Olney et al., 1986). It has been suggested that the time course and morphology of neuronal degeneration in photochemically lesioned retinas is similar to that induced by exposing this organ to large concentrations of glutamate (Mosinger and Olney, 1989), and that glutamate receptor antagonists significantly reduce this type of ischemic damage in vitro and in vivo (Olney et al., 1986; Mosinger et al., 1991). However, quantitative data on the extent of this neuronal protection are still sparse, and in this study we tested several excitatory amino acid receptor antagonists by quantitative evaluation of ischemia-induced damage of the retinal neurons which use acetylcholine or γ-aminobutyric acid (GABA) (Moroni et al., 1993).

2. Materials and methods

2.1. Photothrombotic lesions of the retina

Rose bengal (4,5,6,7-tetrachloro-2',4',5',7-tetraiodofluorescein sodium salt; 80 mg/kg) was injected into the exposed femoral vein of halothane (1.5% in air) anesthetized albino rats as previously described (Moroni et al., 1993). One of their eyes was then illuminated with cold light emitting from a lamp (Osram HLX 150 W) connected to a Nikon fiber optic apparatus filtered at 560 nm (Kodack No. 15 filter). This illumination lasted for 6 min. Either vehicle (5 μl) or an excitatory amino acid antagonist solution was then injected into the vitreous of the lesioned eye by using a Hamilton syringe (701 N) guided by a micromanipulator. The animals were then replaced in their cages. After 2 days the rats were decapitated and both ocular bulbs were surgically removed. The cornea was excised from the removed bulbs and the remaining lens, vitreous and iris were carefully separated from the retinas and discarded. The latter were placed in 0.5 ml of a solution containing EDTA sodium salt (10 mM, pH 7.4) and Triton X-100 (1% of a 10% solution in water) and were homogenized. Previous experiments have shown that rose bengal injection without illumination of the eye does not decrease the retinal marker enzymes (Moroni et al., 1993); therefore, the non-illuminated eye was used as a control and assumed to contain 100% enzymatic activities.

2.2. Measurements of choline-acetyltransferase and glutamic acid decarboxylase activity

Choline-acetyltransferase activity (a marker enzyme for cholinergic amacrine cells) was measured in the homogenate using the method previously described by Fonnum (1975). This method consists of an evaluation of the ability of retinal homogenates to synthesize labeled [^{14}C]acetylcholine from the [^{14}C]acetyl-CoA. Glutamic acid decarboxylase (a marker enzyme for GABAergic neurons) was assayed in the same homogenates used for choline-acetyltransferase measurements using the method previously described by Baxter (1972). The evaluation of this enzymatic activity is based on the measurement of [^{14}C]O_2 formed from the enzyme-induced decarboxylation of [^{14}C]glutamate.

2.3. Materials

[^{14}C]glutamate (55 mCi/mmol) and [^{14}C]acetyl-CoA (58 mCi/mmol) were obtained from Amersham (Amity PG, Italy); acetyl-CoA was purchased from Boehringer Mannheim Biochemica (Milano, Italy); rose bengal was from Sigma Chemical (St. Louis, USA). D-AP5 (D-2-amino-5-phosphonopentanoic acid) was from Tocris Neuramin (Bristol, UK) and MK 801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate) was from RBI (Natick, USA). NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline) was supplied by Dr. M. J. Sheardown (Novo-Nordisk CNS Division, Soeborg, Denmark) and GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5*H*-2,3-benzodiazepine-HCl) by Dr. Tarnawa (Budapest, Hungary). 7-Cl-Thiokynurenic acid and thiokynurenic acid were synthesized by Prof. Pellicciari at the University of Perugia (Italy). Other chemicals were purchased from Merck (Darmstadt, Germany).

Male Wistar rats weighing 200–250 g were obtained from Nossan (Milan, Italy).

2.4. Statistical analysis

Statistical evaluation of the data was performed by ANOVA followed by Dunnett's test. The criterion for significance was $P < 0.05$.

3. Results

3.1. Effects of the photothrombotic lesion

The injection of rose bengal followed by intense illumunation of one eye for 6 min caused diffuse swelling of the inner nuclear and of the inner plexiform layers of the illuminated retina, whose blood vessels were enlarged and occluded by thrombotic material. The inner nuclear and ganglion cell layers had numerous neurons with a swollen cytoplasm and pyknotic nuclei. Severe swelling was present in most of the fibers extending from the inner plexiform layer to the inner limitating membrane. No significant damage was

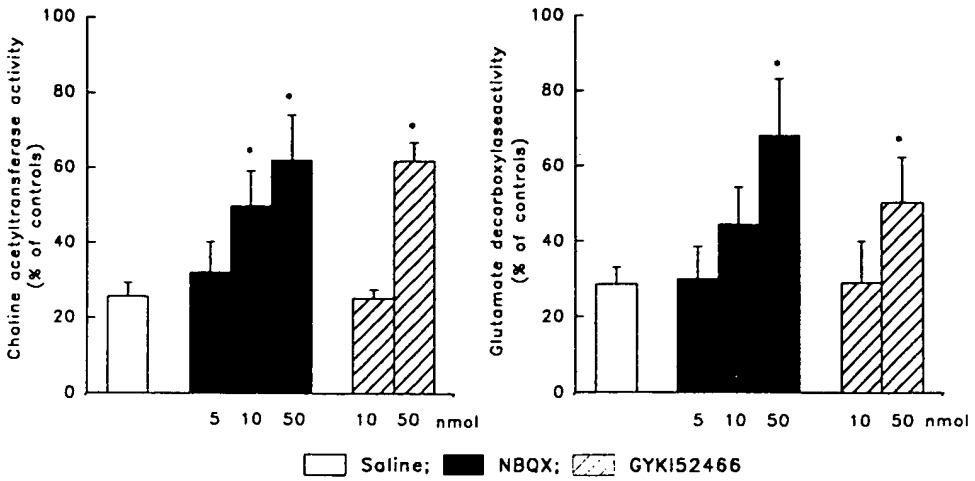

Fig. 1. Effects of AMPA receptor antagonists. The rats were injected with rose bengal (80 mg/kg i.v.) and then one of their eyes was exposed to intense illumination for 6 min (see Methods for experimental details). Sterile solution (5 μl) containing vehicle or different doses of NBQX (5–10–50 nmol), a competitive AMPA receptor antagonist, or of GYKI-52466 (10–50 nmol), a non-competitive AMPA receptor antagonist, was injected intravitreally immediately after light exposure. The animals were killed 2 days later and choline-acetyltransferase and glutamate decarboxylase activities were measured in the retinas as described in Methods. Each column represents the mean percentage of choline-acetyltransferase or glutamate decarboxylase activity present in the lesioned retina as compared with the respective controlateral organ used as control and was calculated from at least seven animals per group. Vertical bars are S.E. In the non-lesioned retinas choline-acetyltransferase activity was 49.9 ± 3.3 nmol/h per retina and glutamic acid decarboxylase activity was 183.6 ± 12 nmol/h per retina (means ± S.E. of at least 120 retinas). * $P < 0.05$ vs. vehicle-injected, lesioned retinas.

observed in the unilluminated retina (Moroni et al., 1993). These histopathological changes were present 4 h after the lesion and were associated with a time-dependent decrease of the activity of the marker enzymes, choline-acetyltransferase and glutamic acid decarboxylase (Moroni et al., 1993). Two days after the lesion, choline-acetyltransferase activity decreased by 75% (from 48 ± 5 in control unlesioned retinas to 12 ± 3 nmol/h per retina in lesioned eyes in which 5 μl of vehicle were injected into the vitreous immediately after exposure to light; means ± S.E. of at least 30 rats) and glutamic acid decarboxylase activity decreased by 72% (from 175 ± 15 in control unlesioned retinas to 49 ± 6 nmol/h per retina in lesioned vehicle-treated eyes; mean ± S.E. of at least 30 rats; see Figs.).

3.2. Effects of competitive and non-competitive AMPA receptor antagonists

The intravitreal injection of NBQX, a prototype competitive antagonist of AMPA receptors (Sheardown et al., 1990), given immediately after the exposure to light, reduced the damage of the cholinergic

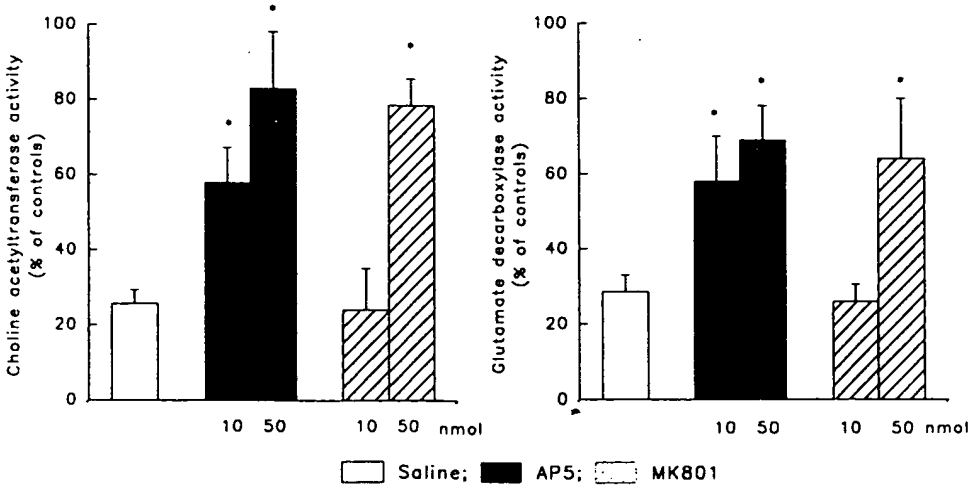

Fig. 2. Effects of intravitreal injection of NMDA receptor antagonists. In these experiments competitive (D-AP5) and non-competitive (MK801) NMDA receptor antagonists were injected intravitreally at the reported doses. See Fig. 1.

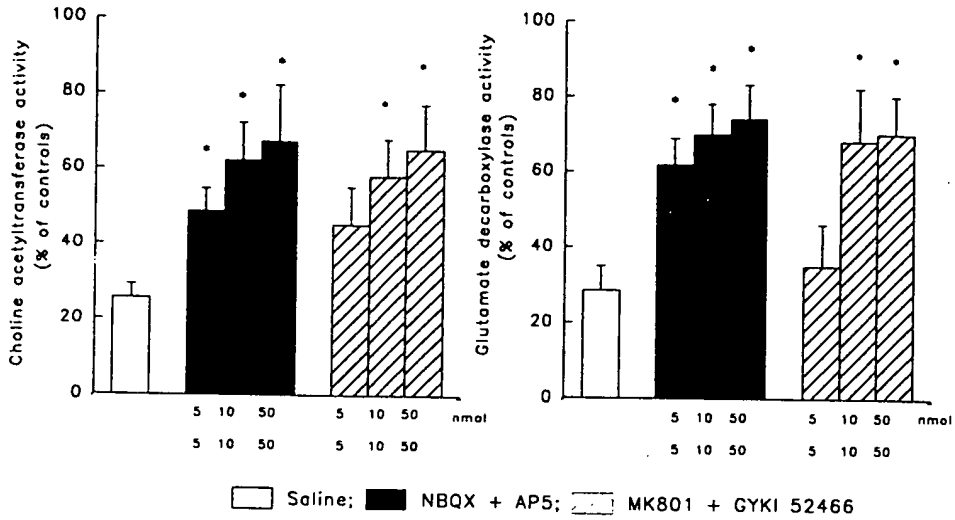

Fig. 3. Effects of the simultaneous intravitreal injection of NMDA and AMPA receptor antagonists. In these experiments combinations of competitive (NBQX + D-AP5) or non-competitive (GYKI 52466 + MK801) NMDA and AMPA receptor antagonists were injected intravitreally at the reported doses. See Fig. 1.

and GABAergic retinal neurons induced by photothrombosis. When 50 nmol of NBQX was injected, the loss of choline-acetyltransferase activity was significantly reduced from 75 ± 5% to 38 ± 10% and that of glutamic acid decarboxylase activity from 72 ± 8% to 32 ± 8% (see Fig. 1). Solubility problems prevented the use of higher doses.

The non-competitive AMPA receptor antagonist, GYKI 52466 (Tarnawa et al., 1989; Donevan and Rogawski, 1993), significantly reduced neuronal damage when injected at 50 nmol (from 75 ± 5 to 39 ± 4% for the choline-acetyltransferase activity and from 72 ± 8% to 50 ± 9% for the glutamic acid decarboxylase activity), but was not active at 10 nmol (see Fig. 1).

3.3. Effects of competitive and non-competitive NMDA receptor antagonists

Fig. 2 shows that a reduction of NMDA receptor ion channel function, obtained by injecting either the competitive antagonist, D-AP5 (Watkins et al., 1990), or the use-dependent ion channel blocker, MK801 (Wong et al., 1986), into the vitreous, protected cholinergic and GABAergic retinal neurons from the photothrombosis-induced lesion. It was interesting to observe that MK801, which is particularly potent in vitro or after systemic administration (Olney et al., 1987; Gill et al., 1987), failed to protect at a dose of 10 nmol and was therefore less potent than D-AP5. Probably MK801,

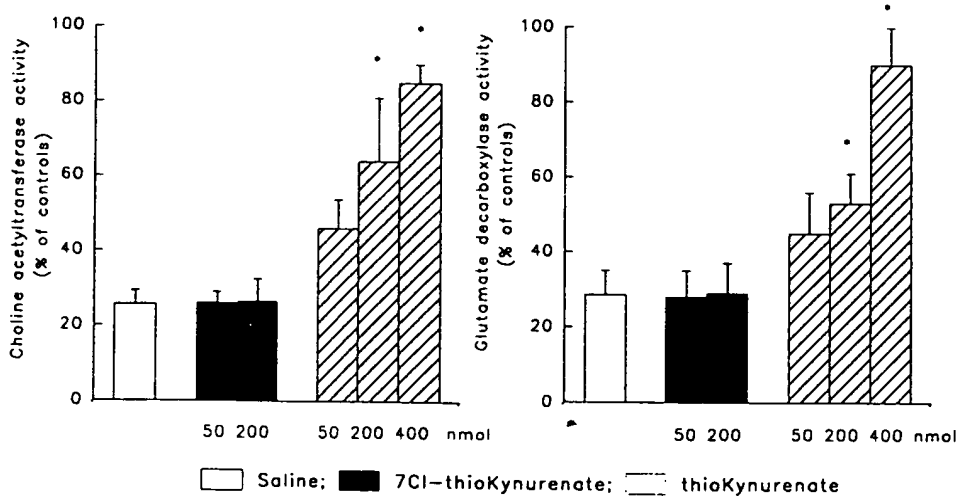

Fig. 4. Effects of intravitreal injection of thiokynurenates. A selective glycine antagonist (7-Clorothiokynurenate) and a broad spectrum antagonist of the glutamate receptors (thiokynurenate) were used at the reported doses. See Fig. 1.

contrary to D-AP5, may diffuse outside of the ocular bulb and this may reduce its protective activity.

3.4. Effects of the intravitreal injection of both NMDA and AMPA receptor antagonists

Fig. 3 shows that the intravitreal injection of both NMDA and AMPA receptor antagonists provided significant protection against photothrombotic lesions. When relatively low doses of the antagonists were used (5 or 10 nmol), the protection was additive. This additive effect was not observed when higher doses (50 nmol) of the compounds were used.

3.5. Effects of thiokynurenate derivatives

The thiokynurenates are a group of compounds able to antagonize excitatory amino acid receptors and to reduce the lipid peroxidation induced by free radicals (Moroni et al., 1991, 1992). 7Cl-Thiokynurenate is a potent and selective antagonist of the glycine recognition site present on the NMDA receptor complex, while thiokynurenate, like kynurenate, is a broad spectrum antagonist of both AMPA and NMDA receptors. Fig. 4 shows that relatively high doses of 7Cl-thiokynurenate did not protect against photochemically induced neuronal damage, while thiokynurenate significantly reduced the lesion, in a dose-dependent manner. The maximum effect obtained with thiokynurenate was similar to that obtained when both AMPA and NMDA receptor antagonists were used.

4. Discussion

The experiments reported here show that direct injection into the vitreal chamber of suitable doses of excitatory amino acid receptor antagonists reduces the ischemic neuronal damage caused by photothrombotic occlusion of retinal vessels. It appears, therefore, that excessive stimulation of glutamate receptors participates in the loss of retinal neurons induced by photothrombosis in a manner resembling that of ischemic brain damage (Rothman and Olney, 1986; Meldrum and Garthwaite, 1990; Choi, 1990). Measurements of choline-acetyltransferase and glutamate decarboxylase activities were used to evaluate the retinal insult quantitatively since neurons using acetylcholine or GABA are particularly abundant in the inner nuclear and ganglion cell layers (Brandon, 1987), the retinal regions which are preferentially damaged in this photothrombotic ischemia model (Mosinger and Olney, 1989; Moroni et al., 1993). Furthermore, the evaluation of these enzymatic activities has been widely used to study excitotoxic brain damage (Schwarcz et al., 1983; Lombardi et al., 1989). Using a phototrombotic occlusion model of retinal vessels and a semiquantitative histological evaluation of retinal neurons, Mosinger et al. (1991) have previously shown that an intravitreal injection of excitatory amino acid receptor antagonists, performed 15 min before the lesion occurred, significantly reduced neuronal damage. This reduction was observed one h after the induction of the phototrombosis and therefore could simply suggest that excitatory amino acid antagonists are able to delay neuronal death. In the present series of observations, photothrombosis-induced neuronal loss was evaluated two days after the lesion occurred and, therefore, it is reasonable to assume that the neuronal protection is indeed permanent. Both NMDA and AMPA receptor antagonists are able to decrease significantly the ischemia-induced loss of retinal choline-acetyltransferase and glutamate decarboxylase activities. This could suggest that the antagonists of the excitatory amino acid receptors allow the neurons to survive with a limited energy supply until the blood returns to the organ. A significant return of blood supply to retinas exposed to a photothrombotic insult has been clearly demonstrated by using fluorescein angiograms made 2–3 days after the lesion (Royster et al., 1988). The protective actions of relatively low doses of AMPA and NMDA receptor antagonists are indeed additive. This additivity is partially lost when higher doses of these compounds are used. Possibly, the complete antagonism of one family of receptors is sufficient to prevent the loss of neurons. Mosinger et al. (1991) described additive protective effects of high doses of CNQX and MK801 1 h after photothrombotic occlusion of retinal vessels. In the present experiments we used NBQX, a compound which is more selective than CNQX as an AMPA antagonist, but which is less potent as a kainate antagonist (Sheardown et al., 1990). In order to obtain optimal protection, the kainate receptors should probably be antagonized and this could explain the difference between the present observations and those reported by Mosinger et al. (1991). We observed that an intravitreal injection of high doses of thiokynurenic acid, a non-specific antagonist of AMPA, kainate and NMDA receptors which can also reduce lipid peroxidation (Moroni et al., 1992), was particularly active in the protection against retinal ischemic damage (see Fig. 4). In contrast, 7Cl-thiokynurenic acid, a selective glycine antagonist (Moroni et al., 1992), completely failed to reduce the ischemia-induced damage up to a relatively high dose (200 nmol). Since this compound has been previously shown to reduce significantly neuronal loss in different brain areas in models of both focal and global ischemia (Chen et al., 1993; Pellegrini-Giampietro et al., 1993), it is possible that the glycine recognition site of the NMDA receptors present in the retina modulates the opening of the channel less efficiently than that of the NMDA receptors present in

neurons located in different areas of the central nervous system. Along this line, it has been demonstrated that the binding of [^{3}H]MK801, used as a functional index of the NMDA receptor complex, is potentiated by glycine to a lesser degree in retinal membranes as compared to membranes obtained from other areas of the central nervous system (Boje et al., 1992). Furthermore, in the retina, glycine does not potentiate NMDA toxicity and actually protects retinal neurons from this type of neuronal damage (Boje et al., 1992). Molecular biology studies show that the NMDA receptor ion channel complex may be assembled from different subunits (Sugihara et al., 1992; Monyer et al., 1992; Hollman et al., 1993) which confer different pharmacological properties on the complex. It is possible that the subunits present in the retina confer low sensitivity to glycine modulation of the receptor. This hypothesis, however, apparently does not explain data obtained in a different laboratory in which high doses of 7Cl-kynurenic acid (0.8–1.2 μmol), another relatively selective glycine antagonist (Kemp et al., 1988), have been shown to protect retinal neurons against ischemic neuronal damage (Mosinger et al., 1991). In our opinion, these high doses of the compound (which probably reach a concentration above 1 mM in the vitreal chamber) cannot be considered selective for the glycine site and could directly affect the recognition sites for glutamate.

In conclusion, this study demonstrates that competitive and non-competitive excitatory amino acid receptor antagonists, directly injected into the vitreal chamber, prevent the loss of cholinergic and GABAergic retinal cells in a model of photothrombosis-induced ischemia. Glycine antagonists, however, do not protect retinal neurons from ischemic retinal damage.

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Activation of Class II or III Metabotropic Glutamate Receptors Protects Cultured Cortical Neurons Against Excitotoxic Degeneration

Exhibit K (10/644,645)

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Keywords: excitotoxicity, metabotropic receptors, neuroprotection, cortical neurons, *N*-methyl-D-aspartate, kainate

Abstract

Trans-1-aminocyclopentane-1,3-dicarboxylic acid, a mixed agonist of all metabotropic glutamate receptor (mGluR) subtypes, is known to produce either neurotoxic or neuroprotective effects. We have therefore hypothesized that individual mGluR subtypes differentially affect neurodegenerative processes. Selective agonists of subtypes which belong to mGluR class II or III, such as (2$_S$,1'$_R$,2'$_R$,3'$_R$)-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV) (specific for subtypes mGluR2 or 3) or L-2-amino-4-phosphonobutanoate and L-serine-*O*-phosphate (specific for subtypes mGluR4, 6 or 7), were highly potent and efficacious in protecting cultured cortical neurons against toxicity induced by either a transient exposure to *N*-methyl-D-aspartate (NMDA) or a prolonged exposure to kainate. In contrast, agonists that preferentially activate class I mGluR subtypes (mGluR1 or 5), such as quisqualate or *trans*-azetidine-2,3-dicarboxylic acid, were inactive. DCG-IV was still neuroprotective when applied to cultures after the toxic pulse with NMDA. This delayed rescue effect was associated with a reduction in the release of endogenous glutamate, a process that contributes to the maturation of neuronal damage. We conclude that agonists of class II or III mGluRs are of potential interest in the experimental therapy of acute or chronic neurodegenerative disorders.

Introduction

Excitatory amino acid receptors are either linked to ion channels ('ionotropic receptors'; iGluRs) or coupled to membrane enzymes, which regulate the concentration of intracellular second messengers ('metabotropic receptors'; mGluRs) (for a review, see Nakanishi, 1992). While the role of iGluRs in the mechanisms of acute and chronic neuronal degeneration is well established (Choi, 1991), the role of mGluRs is still controversial. Activation of mGluRs by the selective agonist *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), or its active isomer 1$_S$,3$_R$-ACPD, produces either neurotoxic or neuroprotective effects depending on the experimental paradigm of toxicity. ACPD attenuates excitotoxic neuronal death in cultured cortical or cerebellar neurons (Koh *et al.*, 1991; Pizzi *et al.*, 1993; Schwarz *et al.*, 1993; cf. Aleppo *et al.*, 1992), and protects retinal neurons against ischaemic damage (Siliprandi *et al.*, 1992). However, local or intracerebroventricular infusion of ACPD induces degeneration of striatal and hippocampal neurons (McDonald and Schoepp, 1992; Olney *et al.*, 1993; Schoepp *et al.*, 1993). The heterogeneity of these effects may be explained by the ability of ACPD to activate various mGluR subtypes, providing that each individual subtype differentially affects neuronal degeneration. mGluRs form a family of at least seven subtypes (mGluR1–7) which have been subdivided into three major classes on the basis of their structural homology and transduction pathway. Class I includes mGluR1 and 5; class II includes mGluR2 and 3; and class III includes mGluR4, 6 and 7 (Nakanishi, 1992; Okamoto *et al.*, 1994; Saugstadt *et al.*, 1994). In transfected cells, mGluR1 and 5 are coupled to polyphosphoinositide (PPI) hydrolysis and their activation generates the intracellular messengers inositol-1,4,5-trisphosphate and diacylglycerol. In addition, activation of mGluR1 (but not mGluR5) leads to an enhanced formation of cyclic AMP (cAMP) and arachidonic acid (Abe *et al.*, 1992; Aramori and Nakanishi, 1992; Houamed *et al.*, 1991; Masu *et al.*, 1991). Members of class II or III of mGluR subtypes (mGluR2–4, 6 and 7) are negatively linked to adenylyl cyclase activity through a pertussis toxin-sensitive GTP-binding protein (Nakanishi, 1992; Tanabe *et al.*, 1992; Okamoto *et al.*, 1994; Saugstadt *et al.*, 1994). The identification of new selective agonists helps dissect the role of individual mGluR subtypes in their native environment. The dicarboxycyclopropylglycine derivative, DCG-IV, behaves as a highly

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potent and selective agonist of mGluR2 and 3, with no substantial activity at mGluR1 or 4 (Hayashi *et al.*, 1993; Ishida *et al.*, 1993; Ohfune *et al.*, 1993), whereas L-2-amino-4-phosphonobutanoate (L-AP4) and L-serine-*O*-phosphate (L-SOP) selectively activate mGluR4, 6 and 7 (Nakanishi, 1992; Tanabe *et al.*, 1993; Saugstadt *et al.*, 1994). *Trans*-azetidine-2,3-dicarboxylic acid (t-ADA) activates mGluRs coupled to PPI hydrolysis (Kozikowski *et al.*, 1993).

We have tested the action of these drugs on neuronal degeneration induced by transient exposure to *N*-methyl-D-aspartate (NMDA) or prolonged exposure to kainate or S-α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) in cultured cortical neurons grown on a monolayer of astrocytes.

Materials and methods

Cortical cell culture

Mixed cortical cell cultures containing both neurons and glia were prepared from fetal mice at 14–17 days of gestation, as described previously (Choi *et al.*, 1987; Rose *et al.*, 1993). Briefly, dissociated cortical cells were plated in 15 mm multiwell vessels (Falcon Primaria, Lincoln Park, NJ) on a layer of confluent glial cells (7–14 days *in vitro*), using a plating medium of Eagle's Minimal Essential Medium (MEM–Eagle's salts, supplied glutamine free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM) and glucose (final concentration 21 mM). Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere. After 3–5 days *in vitro*, non-neuronal cell division was halted by 1–3 days exposure to 10 μM cytosine arabinoside, and cultures were shifted to a maintenance medium identical to plating medium, but lacking fetal serum. Subsequent partial medium replacement was carried out twice a week. Only mature cultures (13–16 days *in vitro*) were used for the experiments.

Glial cell culture

Glial cell cultures were prepared as described previously (Rose *et al.*, 1993) from postnatal mice (1–3 days after birth). Dissociated cortical cells were grown in 15-mm multiwell vessels (Falcon Primaria) using a plating medium of Eagle's salts supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM) and glucose (final concentration 21 mM) (MEM). Cultures were kept at 37°C in a humidified CO_2 atmosphere until they reached confluence (7–14 days *in vitro*). Confluent cultures were then used for the experiments or as a support for mixed cultures.

Pure neuronal culture

Pure neuronal cell cultures were prepared from fetal mice after 14 days of gestation (Choi *et al.*, 1987) with the following modifications. Dissociated cortical cells were plated on 15 mm multiwell vessels (Falcon Primaria) previously coated with poly-D-lysine (100 μg/ml) and laminin (4 μg/ml) using a plating medium of MEM. After 24–48 h a 1/2 exchange of the medium was performed with a glial-conditioned medium (GCM), followed by addition of 3 μM cytosine arabinoside. GCM was prepared from astrocytes grown in 75 cm^2 flasks (Falcon Primaria); after glial cultures reached confluence, the plating medium was changed to fresh MEM lacking serum, and harvested after 2 weeks. Pure neuronal cultures were then kept at 37°C in a humidified CO_2 atmosphere until mature (10–14 days *in vitro*). At this stage, the contamination of glial fibrillary acidic protein-positive cells is only 0.5%, whereas the remaining cells are all neuron-specific enolase-positive.

Exposure to excitatory amino acids

Brief exposure to NMDA (10 min), in the presence or absence of mGluR agonists, was carried out in mixed cortical cultures at room temperature in a HEPES-buffered salt solution (HBSS) containing (in mM): NaCl, 120; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 20; glucose, 15. After 10 min the drugs were washed out and cultures were incubated at 37°C for the following 24 h in medium stock (MS) (Eagle's MEM supplemented with 15.8 mM $NaHCO_3$ and glucose <25 mM). In some experiments, mGluR agonists, or the NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801, 10 μM), were added at different times following the NMDA washout and kept for the 24 h of incubation.

Exposure to kainate or AMPA was carried out at 37°C in MS, in the presence or absence of mGluR agonists (and/or antagonists) or of the kainate/AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM), for 24 h. The NMDA receptor antagonist, MK-801 (10 μM), was present in the incubation medium throughout the experiment to prevent a secondary activation of NMDA receptors by endogenously released glutamate. Little or no cortical cell damage was produced by any of these protocols if excitatory amino acids were omitted.

Assessment of neuronal injury

Neuronal injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at 100–400×, 1 day after the insult when the process of cell death was largely complete. Neuronal damage was quantitatively assessed in all experiments by estimation of dead neurons by trypan blue staining. Stained neurons were counted from three random fields per well. To determine the potency of mGluR agonists as neuroprotectants, neuronal injury was also assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged or destroyed cells into the extracellular medium 1 day after the addition of excitotoxins, as described by Koh and Choi (1987). As results obtained with cell count or measurement of LDH activity were similar (see legend to Fig. 1), only the former method was utilized in further experiments.

Measurement of PPI hydrolysis

Stimulation of PPI hydrolysis was measured as follows: mixed cortical cultures or pure glial cultures were incubated for 18 h with 0.5 μCi of myo-[2-^{3}H]inositol (NEN-DuPont, Milan, Italy; sp. act. 16.5 Ci/mmol) for the labelling of inositol phospholipids. Then, cultures were shifted in Krebs–Henseleit buffer containing 10 μM MK-801, and stimulated with mGluR agonists in the presence of 10 mM LiCl for 20 min. The reaction was stopped by adding cultures to ice-cold methanol/water (1:1). The cells were harvested, and the suspension was added to methanol/chloroform/water (1:1:1). Samples were centrifuged at low speed for 5 min to accelerate phase separation. [^{3}H]Inositolmonophosphate ([^{3}H]InsP) was separated by anion-exchange chromatography as described previously (Nicoletti *et al.*, 1986), and radioactivity was measured by scintillation spectrometry.

Measurement of cAMP formation

Mixed cortical cultures, pure neuronal and pure glial cultures were shifted into MS containing 10 μM MK-801, and incubated for 15 min in the presence of forskolin and/or mGluR agonists. The incubation was terminated by the addition of 0.4 N $HClO_4$ after complete removal of the medium. Cells were scraped and the suspension was added to K_2CO_3 (0.4 N final) and centrifuged at low speed. cAMP

concentration in the supernatant was quantified by a commercially available kit (NEN-DuPont, NEC 033).

HPLC quantitation of extracellular glutamate

Aliquots of the culture bathing medium were collected 1 h after the end of the NMDA pulse and, after pre-column derivatization with an equal volume of *o*-phthaldialdehyde/mercaptoethanol reagent, 25 µl of the derivatized sample was injected onto the HPLC. Glutamate was quantified by a modification of the method of Lindroth and Mopper (1979). Briefly, samples were separated on a Waters Pico-Tag™ column, using a gradient (beginning 4 min after injection) from 100% 0.1 M potassium acetate, pH 7.1/methanol (80:20) to 100% methanol/0.1 M potassium acetate, pH 7.1 (80:20) over 20 min, and fluorescent detection. Commercial standards allowed positive identification and quantitation of amino acids by peak areas.

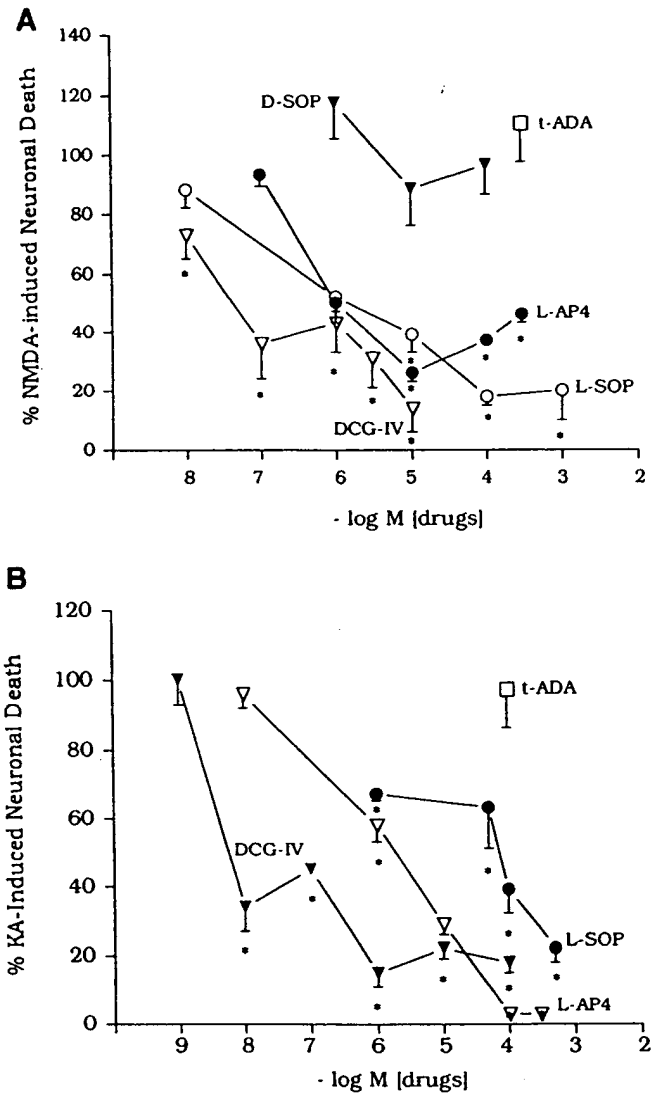

FIG. 1. Concentration–protection relationship of selective agonists of mGluR2 or 3 (DCG-IV) or mGluR4, 6 or 7 (L-AP4 and L-SOP) against NMDA (A) or kainate (B) toxicity in mixed cortical cultures. Data refer to the number of cells stained with trypan blue (mean±SEM) and are expressed as per cent of NMDA (100 µM)- or kainate (60 µM)-induced neuronal degeneration. Values were calculated from 6–24 individual determinations from 2–6 independent experiments. $^*P < 0.01$ (one-way ANOVA + Fisher PLSD test), as compared with values obtained in the absence of mGluR agonists. Extracellular LDH activity was measured in parallel in all the conditions we have examined. LDH activity (expressed as mOD/min) was 29±7 in control cultures; 230±16 in cultures exposed to NMDA; and 315±24 in cultures exposed to kainate. Concentration curves obtained with mGluR2 agonists were similar to those obtained by counting the number of cells stained with trypan blue.

Materials

NMDA, kainate, L-SOP, D-SOP, forskolin and N^6-2′-*O*-dibutyryl-adenosine-3′,5′-cyclic monophosphate (dbcAMP) were obtained from Sigma (St Louis, MO); AMPA, quisqualate, $1_S,3_R$-ACPD, L-AP4, (RS)-α-methyl-4-carboxyphenyl-glycine, and CNQX were purchased from Tocris Neuramin (Essex, UK). MK-801 was obtained from RBI (Natick, MA). DCG-IV, L-2-carboxycyclopropylglycine (L-CCG-I) and t-ADA were synthesized and kindly provided by Dr H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Japan), Dr R. Pellicciari (Institute of Pharmacology and Chemistry, University of Perugia, Italy) and Dr A. P. Kozikowski (Neurochem. Res., Mayo Foundation, Jacksonville) respectively.

Results

Toxicity induced by a transient exposure to NMDA

A 10 min exposure to NMDA induced neuronal degeneration in mixed cultured cells, as reflected by both an increased number of trypan blue-stained neurons and an enhanced LDH release. The action of NMDA was concentration-dependent (EC_{50} value = 60 µM) and was totally antagonized by 10 µM MK-801. At maximal concentrations (>150 µM) NMDA killed the entire neuronal population, without damaging astrocytes either in mixed or in pure glial cultures. We have routinely used a submaximal concentration of NMDA (100 µM), which led to necrotic degeneration of 77±8% of cortical neurons in standard experiments (not shown). mGluR agonists (co-applied with NMDA) attenuated NMDA-induced neuronal degeneration, with the following rank order of potency (EC_{50} in parentheses): DCG-IV > L-AP4 (1 µM) = L-SOP (2 µM) = L-CCG-I

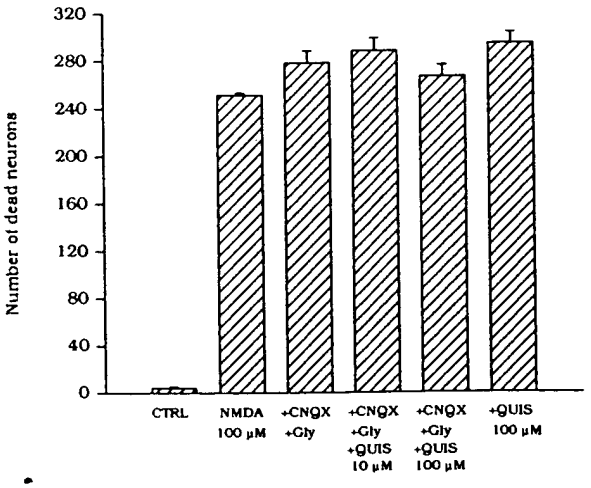

FIG. 2. Quisqualate fails to attenuate NMDA toxicity, when applied alone or in combination with CNQX or glycine. A 10-min pulse with quisqualate without NMDA did not affect neuronal viability. Values (mean±SEM) refer to the number of neurons stained with trypan blue from three random microscopic fields per well, and were calculated from eight individual determinations from two independent experiments.

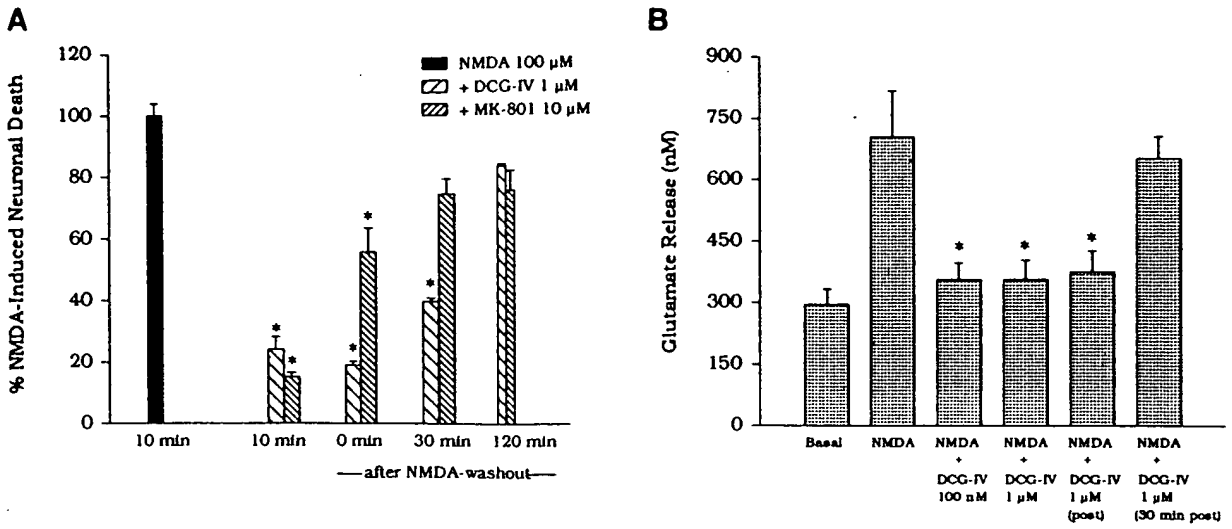

FIG. 3. (A) Neuroprotective action of DCG-IV or MK-801 applied during, immediately, 30 or 120 min after the NMDA pulse. Values (mean±SEM) refer to the number of neurons stained with trypan blue and were calculated from 8–12 individual determinations from three independent experiments. (B) Extracellular concentration of glutamate in cultures exposed to NMDA in the absence or presence of DCG-IV. DCG-IV was applied either during, immediately after (post) or 30 min after (30 min post) the NMDA pulse. Glutamate levels were measured 1 h after the excitotoxic pulse. $^*P < 0.01$ (one-way ANOVA + Fisher PLSD), when compared with values obtained with NMDA alone.

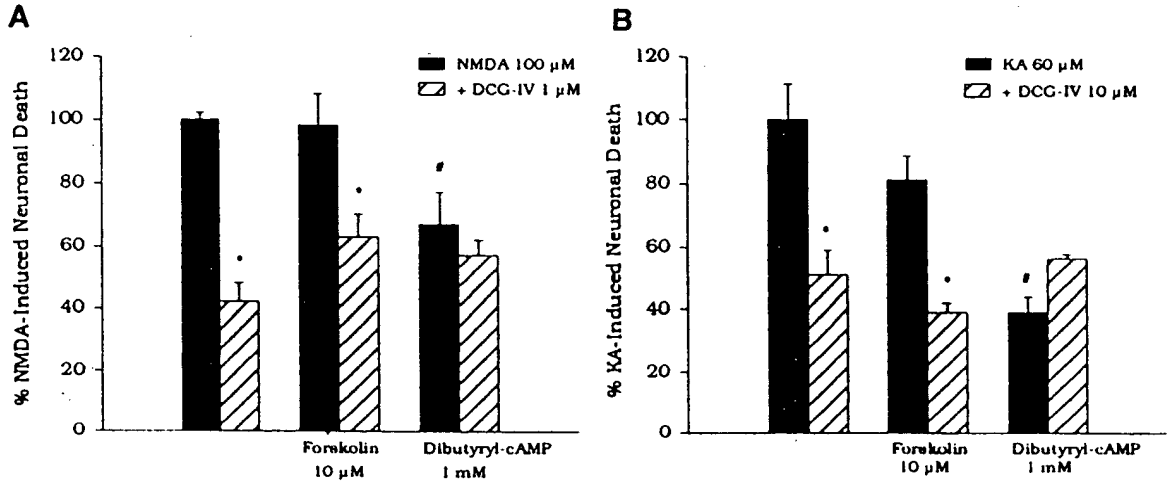

FIG. 4. Neuroprotective activity of DCG-IV against (A) NMDA- or (B) kainate (KA)-induced toxicity in mixed cortical cultures incubated in the absence or presence of forskolin or dbcAMP (both co-incubated with DCG-IV and NMDA or kainate). Values (mean±SEM) refer to the number of neurons stained with trypan blue and were calculated from eight individual determinations from two independent experiments. Neither forskolin nor dbcAMP influenced *per se* neuronal viability (not shown). $^*P < 0.01$ (one-way ANOVA + Fisher PLSD) when compared with the respective value obtained in the absence of DCG-IV; $^\#P < 0.01$ (one-way ANOVA + Fisher PLSD) when compared with the value obtained with NMDA or kainate alone.

(3 μM) >> $1_S,3_R$-ACPD (>300 μM). The IC_{50} value of DCG-IV could not be easily determined because the curve had the tendency to be biphasic. DCG-IV was however substantially active at concentrations as low as 100 nM. D-SOP and t-ADA were inactive (up to 300 μM) (Fig. 1A; Bruno *et al.*, 1994). Quisqualate (10 or 100 μM) was inactive against NMDA toxicity when applied alone or in combination with 10 μM CNQX (to prevent activation of AMPA receptors) and 20 μM glycine (to overcome the inhibitory action of CNQX at the glycine site of NMDA receptors) (Fig. 2). None of the above mentioned drugs influenced *per se* neuronal viability (quisqualate was toxic but only when applied for 24 h in the absence of CNQX; not shown).

DCG-IV substantially attenuated NMDA toxicity even when applied immediately or 30 min after the NMDA pulse, under conditions in which MK-801 lost most of its neuroprotective activity. The neuroprotective activity of DCG-IV applied immediately after the pulse was not different from that observed when the drug was combined with NMDA. Both DCG-IV and MK-801 were virtually inactive when applied 2 h after the NMDA pulse (Fig. 3A). To shed light on the delayed rescue of cortical neurons by DCG-IV, we

TABLE 1. mGluR agonists fail to protect cultured cortical neurons against AMPA-induced toxicity

	% AMPA-induced toxicity
DCG-IV 10 μM	75±16
L-CCG-I 100 μM	76±2.5
L-AP4 100 μM	94±8
L-SOP 100 μM	78±2
$1_S,3_R$-ACPD 300 μM	87±4.3
CNQX 30 μM	2±0.2

Values (means±SEM) are expressed as per cent of AMPA-induced toxicity and were calculated from the means of 8–12 determinations from 2–3 independent experiments.

measured the amount of glutamate released into the medium during the development of neuronal degeneration. The extracellular concentration of glutamate significantly increased 60 min after the NMDA pulse. This increase was totally prevented by DCG-IV applied either during or immediately after the NMDA pulse. DCG-IV was however inactive when applied 30 min after the pulse (Fig. 3B).

To investigate the role of cAMP in the neuroprotective action of DCG-IV, we have performed experiments in the presence of forskolin (a potent activator of adenylyl cyclase activity) or dbcAMP (a membrane permeable analogue of cAMP). While DCG-IV was still neuroprotective in the presence of forskolin, the action of DCG-IV was no longer visible in cultures exposed to dbcAMP, which, however, exhibited an intrinsic neuroprotective activity (Fig. 4A).

Toxicity induced by a prolonged exposure to kainate or AMPA

Cultures exposed to kainate for 24 h (in the presence of 10 μM MK-801) exhibited extensive neuronal degeneration. The action of kainate was concentration-dependent (EC_{50} value = 40 μM) and was antagonized by 30 μM CNQX. Maximal concentrations of kainate (>150 μM) killed the entire neuronal population without damaging the underlying monolayer of astrocytes. At the concentration we have used (60 μM), kainate led to necrotic degeneration of 62±7% of cultured cortical neurons. mGluR agonists (co-applied with kainate) attenuated kainate-induced toxicity with the following rank order of potency (EC_{50} in parentheses): DCG-IV (4 nM) >> L-CCG-I (2 μM) > L-AP4 (10 μM) = $1_S,3_R$-ACPD (17 μM) > L-SOP (73 μM). Also in this case, the concentration–response curve with DCG-IV had the tendency to be biphasic. t-ADA was inactive up to 300 μM (Fig. 1B).

The neuroprotective action of DCG-IV was not influenced by forskolin and was obliterated in the presence of dbcAMP, which was neuroprotective *per se* (Fig. 4B).

In contrast to the other paradigms of neuronal degeneration, toxicity induced by 24-h exposure to AMPA was resistant to DCG-IV (10 nM–10 μM), L-CCG-I (100 μM), L-AP4 (1–300 μM), L-SOP (100 μM) or $1_S,3_R$-ACPD (10–300 μM), although it was totally prevented by CNQX (Table 1).

Stimulation of PPI hydrolysis

In mixed cultures, quisqualate was the most potent mGluR agonist in stimulating [^{3}H]InsP formation, followed by L-CCG-I and $1_S,3_R$-ACPD and then by t-ADA, which exhibited the lowest potency (Fig. 5). All these drugs exhibited the same efficacy in stimulating [^{3}H]InsP formation, with a maximal stimulation of ~2-fold above basal. DCG-IV was inactive *per se* and did not amplify quisqualate-stimulated PPI hydrolysis as it does in brain slices (Genazzani *et al.*, 1994).

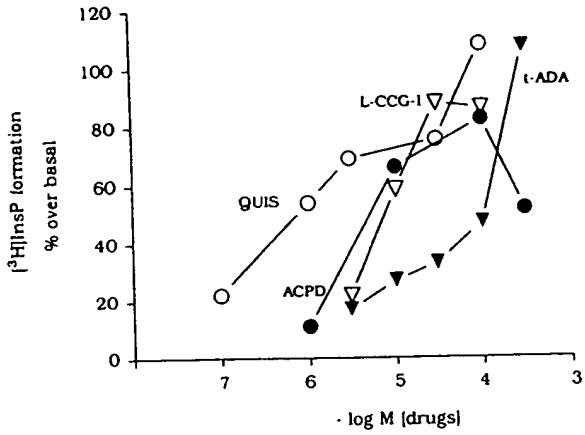

FIG. 5. Stimulation of PPI hydrolysis by mGluR agonists in mixed cortical cultures. Values are expressed as per cent above basal and were calculated from the mean of 4–12 individual determinations from 1–3 independent experiments. SD was always <10% of the mean values. Basal [^{3}H]InsP formation was 6200±130 d.p.m./well (n = 12).

TABLE 2. Stimulation of cAMP formation by forskolin in mixed, glial or neuronal cortical cultures incubated in the absence or presence of mGluR agonists

	cAMP formation (pmol/well)		
	Mixed cultures	Glial cultures	Neuronal cultures
Bas	47±3*	34±6*	42±3.5*
Forskokin 10 μM	295±16	128±19	77±3
+ DCG-IV 100 nM	n.d.	n.d.	43±3.5*
+ DCG-IV 10 μM	300±28	146±14	37±2.5*
+ L-CCG-I 1 μM	n.d.	n.d.	48±3.5*
+ L-CCG-I 100 μM	228±28	n.d.	31±3*
+ ACPD 30 μM	n.d.	n.d	50±2*
+ ACPD 100 μM	269±14	124±11	37±6.5*
+ L-AP4 100 μM	287±30	136±16	31±2*
+ L-SOP 100 μM	298±48	n.d.	n.d.
+ t-ADA 300 μM	296±48	n.d.	82±4

Values are means±SEM of 4–12 individual determinations from 1–3 independent experiments. *P < 0.01 (one-way ANOVA + Fisher PLSD) when compared with values obtained with forskolin alone. None of the mGluR agonists influenced *per se* intracellular cAMP levels.

None of the mGluR agonists increased PPI hydrolysis in pure cultures of cortical astrocytes (not shown).

Inhibition of forskolin-stimulated cAMP formation

In both mixed cortical cultures or pure cultures of cortical astrocytes, a 15-min exposure to forskolin (10 μM) induced a substantial increase in cAMP formation (528 and 276% above basal respectively). This increase was not influenced by any of the mGluR agonists we have tested, including DCG-IV, L-CCG-I, L-AP4, L-SOP, $1_S,3_R$-ACPD and t-ADA (Table 2). To establish whether a potential effect of mGluR agonists in neurons of mixed cultures was obliterated by the robust cAMP response of the underlying astrocytes, we have performed experiments in pure cultures of cortical neurons. In this model, forskolin stimulated cAMP formation to a much lower extent (~79% above basal) and this stimulation was prevented by DCG-IV,

L-CCG-I, L-AP4, $1_S,3_R$-ACPD but not by t-ADA (up to 300 μM) (Table 2).

Discussion

An excitotoxic mechanism has been implicated in a variety of acute and chronic neurodegenerative disorders, which include brain ischaemia, hypoglycaemia, status epilepticus, posttraumatic injury, Huntington's chorea, Alzheimer's disease and parkinsonism (Choi, 1988; Maragos *et al.*, 1987; Young *et al.*, 1988). Hence, the identification of the transsynaptic or intracellular mechanisms underlying excitotoxic neuronal degeneration may have important clinical implications. The role of mGluRs in neuronal degeneration has been so far controversial (for a review see Schoepp and Conn, 1993), because of the lack of specific drugs which can discriminate among the various receptor subtypes. The pharmacology of mGluRs is now enriched with new potent and selective agonists, which bear a cyclopropylglycine motif. While L-CCG-I behaves as a mixed mGluR agonist (albeit preferential for mGluR2 and 3) (Nakanishi, 1992), DCG-IV is highly selective for mGluR2 and 3 (Hayashi *et al.*, 1993; Ishida *et al.*, 1993; Ohfune *et al.*, 1993). DCG-IV was the most potent and efficacious drug we have tested (see also Bruno *et al.*, 1994) in protecting cultured cortical neurons against toxicity induced by either a brief exposure to NMDA or prolonged exposure to kainate. The neuroprotective effect of DCG-IV against NMDA toxicity in cultured cortical cells has been recently confirmed by Buisson *et al.* (1994). We tested whether DCG-IV retained its neuroprotective activity when applied after the induction of the excitotoxic damage. DCG-IV could still rescue a large percentage of cortical neurons when applied immediately or after 30 min, but not 2 h after the NMDA pulse. This 'delayed rescue' effect (Hartley and Choi, 1989) was stronger than that induced by the NMDA receptor antagonist MK-801, which lost part of its neuroprotective activity when applied after the NMDA pulse. The delayed rescue effect of DCG-IV was associated with a reduction in the release (or leakage) of endogenous glutamate, a process which occurs early after the NMDA pulse and contributes to the progression of neuronal damage (Choi, 1992). DCG-IV has been reported to depress synaptic transmission in the spinal cord (Ishida *et al.*, 1993) and to reduce GABA release in the dendro-dendritic synapses between mitral and granule cells of the accessory olfactory bulb, where mGluR2 is presynaptically located (Hayashi *et al.*, 1993). It is therefore possible that activation of presynaptic mGluR2 reduces the release of glutamate from neurons exposed to toxic concentrations of NMDA, and that this mechanism contributes to the neuroprotective activity of DCG-IV. To what extent it can contribute is unclear; other mechanisms should also be involved because DCG-IV retained part of its neuroprotective activity when applied 30 min after the NMDA pulse, a time at which it fails to reduce glutamate release.

A reduced glutamate release can in principle be involved also in the neuroprotective action of $1_S,3_R$-ACPD, which exhibits high affinity for mGluR2/3, and depresses excitatory synaptic transmission in a variety of models (Baskys and Malenka, 1991; Lovinger, 1991; Calabresi *et al.*, 1992). However, $1_S,3_R$-ACPD is known to activate a presynaptic mGluR coupled to PPI hydrolysis, which enhances glutamate release in the presence of arachidonic acid (Herrero *et al.*, 1992). The presence of this receptor (the identity of which is unknown) in cultured cortical neurons may contribute to explain why $1_S,3_R$-ACPD is much less active than DCG-IV as a neuroprotectant.

Interestingly, DCG-IV was even more potent in protecting cultured cortical neurons against kainate toxicity, under conditions in which any secondary activation of NMDA receptors was prevented by the presence of 10 μM MK-801. This effect is consistent with *in vivo* studies showing that DCG-IV reduces both kainate-induced limbic motor seizures and kainate-induced neuronal death in the CA3 region of rat hippocampus (Kwak *et al.*, 1994; Shinozaki *et al.*, 1994). Although activation of mGluR2 or 3 may be relevant for the neuroprotective action of DCG-IV, we cannot exclude the possibility that other mechanisms are involved, because the concentration–response curves with DCG-IV had the tendency to be biphasic when the drug was challenged against both NMDA and kainate toxicity.

L-AP4 and L-SOP, which potently activate mGluR4, 6 and 7 (Nakanishi, 1992; Schwarz *et al.*, 1993; Saugstadt *et al.*, 1994), also exhibited high potency in protecting mixed cultures of cortical neurons against NMDA or kainate toxicity. Their action should be mostly mediated by mGluR7b (one of the two spliced variants of mGluR7; T. Knoepfel, personal communication), the expression of which is more abundant compared to that of mGluR4 in cultured cortical neurons (Bruno *et al.*, in preparation).

Among the possible mechanisms responsible for neuroprotection, one first possibility is that class II or III mGluRs negatively modulate the electrophysiological responses (e.g. membrane depolarization) induced by NMDA or kainate. It has been recently shown that DCG-IV reduces NMDA currents in mixed cortical cultures (Buisson *et al.*, 1994). Although this effect may contribute to the neuroprotective action of DCG-IV against NMDA toxicity (Bruno *et al.*, 1994; Buisson *et al.*, 1994; present data), it cannot explain the 'delayed rescue' effect of DCG-IV, which is much greater than that produced by the potent NMDA receptor antagonist MK-801.

What class II or III mGluRs have in common is that they are all negatively linked to adenylyl cyclase in transfected cells (Nakanishi, 1992; Tanabe *et al.*, 1992). In mixed cortical cultures, none of the mGluR agonists we have tested was able to reduce forskolin-stimulated cAMP formation due to a large background provided by the underlying monolayer of astrocytes (which were unresponsive to mGluR agonists). However, DCG-IV, L-CCG-I, $1_S,3_R$-ACPD, L-AP4 and L-SOP could inhibit forskolin-stimulated cAMP formation in pure neuronal cultures, indicating that class II or III mGluRs are negatively linked to adenylyl cyclase in cortical neurons. By decreasing intracellular cAMP levels, class II or III mGluRs could, in principle, decrease the open time of high threshold voltage-sensitive L-type Ca^{2+} channels (Chetkovich *et al.*, 1991; Goulding *et al.*, 1994) and increase a specific K^+ current (K_{AHP}), which is responsible for after-hyperpolarization and spike accommodation (Gereau IV and Conn, 1994). This would decrease both neuronal excitability and Ca^{2+} influx, thus rendering neurons less vulnerable to the excitotoxic insult. To assess whether a reduction in cAMP was instrumental for the neuroprotective action of mGluR agonists, we tested the neuroprotective activity of DCG-IV in the presence of forskolin (a potent activator of adenylyl cyclase) or dbcAMP (a membrane permeable analogue of cAMP). Whereas forskolin had little or no influence, dbcAMP obliterated the neuroprotective action of DCG-IV (also see Buisson *et al.*, 1994). However, dbcAMP exhibited an intrinsic neuroprotective activity (Mattson and Kater, 1988), which has no apparent explanation, but is difficult to reconcile with the hypothesis that mGluR agonists rescue cortical neurons by reducing the intracellular levels of cAMP. It is possible that native class II or III mGluRs are associated with other transduction pathways, which have not been identified in transfected cells. Both ACPD and L-AP4 are known to influence the activity of a variety of ion channels, including L or N types of voltage-sensitive Ca^{2+} channels (Fagni *et al.*, 1991; Lester and Jahr, 1990; Trombley and Westbrook, 1992). The specific subtypes which mediate these effects are generally

unknown, although in cultured cerebellar granule cells activation of mGluRs has been associated with inhibition of L-type voltage-sensitive Ca^{2+} channels (Fagni *et al.*, 1993). In addition, a specific mGluR with a pharmacological profile typical of mGluR2/3 has been shown to activate a large conductance K^+ channel (BK type) in neurons of cortical amygdala (Shinnik-Gallagher *et al.*, 1994). The possibility that modulation of Ca^{2+} or K^+ channels is involved in the neuroprotective role of specific mGluR subtypes is worth investigating. The lack of neuroprotective activity by quisqualate (a potent agonist of mGluR1 and 5) and t-ADA (which enhanced [^{3}H]InsP formation without affecting the cAMP pathway) suggests that stimulation of PPI hydrolysis does not contribute to the neuroprotective action of mGluR agonists. Selective and potent mGluR antagonists are needed to establish whether the stimulation of PPI hydrolysis by ACPD is potentially neurotoxic and explains the occurrence of neuronal degeneration after intracerebral infusion of ACPD (McDonald and Schoepp, 1992; Olney *et al.*, 1993; Schoepp *et al.*, 1993).

In conclusion, we have shown that selective agonists for mGluR subtypes that are negatively linked to adenylyl cyclase in transfected cells (i.e. mGluR2, 3, 4, 6 and 7) protect cultured cortical neurons against the toxic action of NMDA and kainate. The resistance of AMPA-mediated toxicity to mGluR agonists suggests a different mode of action of AMPA and kainate in inducing neuronal degeneration. Studies in transfected cells show that kainate interacts with receptors not only formed by the proper AMPA receptor subunits (GluR1-4) but also by subunits GluR5, 6, 7 or KA1 or 2. It is possible that only the latter receptors are under the negative influence of class II or III mGluRs. Whether this reflects a differential anatomical compartmentalization of these subunits or is rather in relation to their different permeability to Ca^{2+} ions is unknown at present. Although DCG-IV exhibited the highest potency as a neuroprotective agent and was still active when applied after the excitotoxic insult, its potential use is hampered by its ability to activate NMDA receptors at high concentrations (Ishida *et al.*, 1993; Ohfune *et al.*, 1993). (2_S,$1'_R$,$2'_R$,$3'_R$)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine has been recently introduced as a novel mGluR ligand, which shares the ability of DCG-IV to depress synaptic transmission but does not activate NMDA receptors (Shinozaki, 1993). The synthesis of potent and selective agonists for mGluRs negatively linked to adenylyl cyclase may thus furnish new pharmacological tools for the experimental therapy of acute or chronic neurodegenerative disorders.

Abbreviations

ACPD	*trans*-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	S-α-amino-3-hydroxy-5-methyl-4-isoxale propionate
cAMP	cyclic AMP
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
dbcAMP	N^6-2′-*O*-dibutyryladenosine-3′,5′-cAMP
DCG-IV	(2_S,$1'_R$,$2'_R$,$3'_R$)-2-(2,3-dicarboxycyclopropyl)glycine
GCM	glial-conditioned medium
[^{3}H]InsP	[^{3}H]inositol monophosphate
iGluR	ionotrophic receptors
L-AP4	L-2-amino-4-phosphonobutanoate
L-CCG-I	L-2-carboxycyclopropylglycine
LDH	lactate dehydrogenase
MEM	minimal essential medium
mGluR	metabotropic glutamate receptor
MS	medium stock
NMDA	*N*-methyl-D-aspartate
PPI	polyphosphoinositide
SOP	serine-*O*-phosphate
t-ADA	*trans*-azetidine-2,3-dicarboxylic acid

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Note added in proof

Recently a new mGluR subtype (mGluR8) has been described [reviewed by Pim, J.-P. and Duvoisim, R. (1995) The metabotropic glutamate receptors: structure and function. *Neuropharmacology*, **34**, 1–26].

Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage

Dennis W. Choi Exhibit L (10/644, 645)

An influx of extracellular Ca^{2+}, with subsequent cellular Ca^{2+} overload, can clearly cause certain types of cell death, and has been hypothesized to be a primary etiological event in hypoxic–ischemic neuronal injury. Recently, this hypothesis has acquired new specificity, as hypoxic–ischemic neuronal injury has been linked to the excessive activation of postsynaptic glutamate receptors, and glutamate neurotoxicity itself has been linked to a lethal influx of extracellular Ca^{2+} through cell membrane channels. Available data suggest that channels gated by the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors may be the predominant route of glutamate- or hypoxia-induced lethal Ca^{2+} entry; however, other routes, including L- and N-type voltage-gated Ca^{2+} channels, the Na^+/Ca^{2+} exchanger, and non-specific membrane leak, may also participate. Current efforts to develop an effective therapy for hypoxic–ischemic neuronal injury are appropriately focused on NMDA antagonists; however, it is possible that additional benefit might be gained by combining NMDA antagonists with pharmacological manipulations designed to attenuate Ca^{2+} entry through these other routes.

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For normal function, the cytosol of most cells requires the intricate regulation of Ca^{2+} concentrations in the range of 50–300 nM; yet only a membrane away are both an extracellular Ca^{2+} concentration four orders of magnitude higher, and large intra-organelle Ca^{2+} stores. This precarious situation is the basis for the long-standing hypothesis that hypoxic–ischemic neuronal injury, like several other forms of cellular injury, may be caused in large part by a massive disturbance in Ca^{2+} homeostasis. In the past four years, this hypothesis has acquired fresh specificity, as existing supporting evidence has converged with newer evidence linking hypoxic–ischemic neuronal injury to the excessive activation of postsynaptic glutamate receptors, and the latter to a lethal influx of extracellular Ca^{2+} through cell membrane channels.

Role of extracellular Ca^{2+} in cell injury

An influx of extracellular Ca^{2+}, with subsequent cellular Ca^{2+} overload, can clearly cause certain types of cell death. One example is the 'calcium paradox' phenomenon in myocardium, where cardiac cells exposed transiently to Ca^{2+}-free solution rapidly accumulate a lethal load of Ca^{2+} upon return to Ca^{2+}-containing solution[1]; another is the destruction of cultured hepatocytes by the Ca^{2+} ionophore A23187 (Ref. 2). Removing extracellular Ca^{2+} reduces the injury of cultured hepatocytes[3] and lymphocytes[4] by various membrane-active toxins. Excessive Ca^{2+} influx also appears to contribute to several categories of muscle cell injury induced by excess exposure to a transmitter agonist: (1) cardiac cells by catecholamines[5]; (2) skeletal muscle cells by cholinergic agonists[6]; and (3) invertebrate muscle cells by glutamate[7].

There are probably myriad reasons why sustained elevations in cytosolic Ca^{2+} are toxic, reflecting summated gross disturbances in the many biological processes regulated by Ca^{2+} availability. Some specific events triggered by excess intracellular Ca^{2+} include activation of intracellular proteases and lipases, generation of free radicals, depletion of energy reserves by activation of Ca^{2+}-ATPase, and impairment of mitochondrial oxidative phosphorylation[8].

Evidence implicating Ca^{2+} overload as a pathogenetic mechanism in hypoxic–ischemic neuronal injury has been critically reviewed[9]. Ca^{2+} is probably not a common pathway mediating all ischemic cell death. In certain models of hypoxic–ischemic injury involving non-neuronal cells, there does not appear to be a correlation between cell viability and Ca^{2+} content[8]; convincingly, a rise in cytosolic free Ca^{2+} does not precede injury in hepatocytes exposed to chemical hypoxia[10]. However, with regard to the hypoxic–ischemic injury of central neurons, several observations have supported a primary etiological role for cellular Ca^{2+} overload: (1) hypoxia in cerebral cortex is accompanied by a dramatic decline in extracellular Ca^{2+} to about 10% of control levels[11]; (2) early events in neuronal hypoxia, including transmitter release and liberation of free fatty acids, suggest a rise in intracellular Ca^{2+} (Ref. 9); (3) Ca^{2+} accumulation in hypoxic brain is correlated at the regional[12] and cellular[13] levels with vulnerability to hypoxic injury, and in fact may occur before neurons appear necrotic under the light microscope[9]; (4) hypoxic neuronal injury *in vitro* under low Na^+ conditions is highly Ca^{2+}-dependent[14]; and (5) organic blockers of voltage-dependent Ca^{2+} channels show protective efficacy in some animal models of ischemia[9,15]. As discussed below, this Ca^{2+} hypothesis of hypoxic–ischemic neuronal injury has acquired additional support from the recent establishment of links both between hypoxic–ischemic neuronal injury and glutamate neurotoxicity, and between glutamate neurotoxicity and Ca^{2+}-mediated injury mechanisms.

Glutamate neurotoxicity in hypoxic–ischemic neuronal injury

The neurotoxicity of glutamate (Glu), and to a lesser extent related endogenous excitatory amino acids (EAAs), may play an important role in the pathogenesis of hypoxic or ischemic central neuronal injury[16]. The stage is set by the presence of 10 mM concentrations of Glu in the CNS, concentrations two orders of magnitude higher than those required to damage cortical neurons irreversibly with a 5 min extracellular exposure[17].

The onset of hypoxia is soon followed by neuronal depolarization and increased release of Glu and related compounds from excitatory terminals. In addition, cellular uptake mechanisms for EAAs, which are dependent on the presence of high-energy phosphates, are inactivated by the energy depletion

that accompanies hypoxia. The combination of excessive synaptic release and impaired cellular uptake would be expected to lead to a build-up of EAAs in the synaptic and extracellular space; using intracerebral microdialysis, Benveniste *et al.* measured an eightfold increase in extracellular Glu in the ischemic rat hippocampus[18].

Key evidence supporting the importance of synaptic transmission in hypoxic neuronal injury was provided by *in-vitro* experiments showing that a high concentration of Mg^{2+} reduced the vulnerability of hippocampal neurons to anoxia[19,20]. Attention was targeted specifically on excitatory synaptic transmission by pharmacological studies showing that hypoxic neuronal injury could be attenuated by the administration of Glu antagonists both *in vitro*[21] and *in vivo*[22].

Role of Ca^{2+} in glutamate neurotoxicity

The mechanisms by which excess exposure to Glu and other endogenous EAAs can produce neuronal cell injury are not fully understood, but recent studies have indicated important roles for both extracellular Na^+ and extracellular Ca^{2+}.

In their early work characterizing EAA neurotoxicity, Olney and colleagues noted a general correspondence between the neuroexcitatory and neurotoxic potential of many compounds (but see below), and developed the 'excitotoxicity' hypothesis[23], i.e. that EAA neurotoxicity was a direct consequence of excessive excitatory depolarization, perhaps because of associated impaired ion homeostasis, or depletion of cell energy reserves. This attractive hypothesis was strengthened by their ultrastructural studies, showing that EAAs induced neuronal swelling that was most marked in neuronal dendrites and cell bodies ('dendrosomatotoxic'), sites likely to bear high concentrations of postsynaptic excitatory receptors.

Subsequent *in-vitro* experiments have also supported the existence of an acute excitotoxic mechanism of EAA-induced neuronal injury. Cultured hippocampal neurons[24] and neurons in the isolated chick retina[25] exhibited irreversible acute toxic swelling upon 30 min exposure to EAAs, even in the absence of extracellular Ca^{2+}. Since EAA neuroexcitation is largely mediated by an inward movement of Na^+ ions through chemically activated membrane conductances[26], the excitotoxicity concept predicts that neuronal injury would depend upon the presence of extracellular Na^+. In fact, replacement of extracellular Na^+ with an impermeant cation was found to block this acute toxic swelling[24,25]; similar protection was also achieved by replacing extracellular Cl^- with an impermeant anion, a manoeuver expected to decrease net Na^+ (and water) influx secondarily by limiting availability of an anion partner for this influx. Furthermore, the acute neuronal swelling produced by EAA exposure could be mimicked by other depolarizing agents such as high K^+ or veratridine[24].

However, the rank correlation for EAAs between neuroexcitation and neurotoxicity is not perfect; for example, kainate is intrinsically a potent neuroexcitant[27], but a weak neurotoxin on most cortical neurons[17] dependent on endogenous Glu projections for *in-vivo* neurotoxicity[28]. More direct evidence that acute excitotoxicity cannot explain the entire spectrum of EAA toxicity was provided by observations in murine cortical cell cultures. Although replacement of extracellular Na^+ abolished the acute neuronal swelling seen in these cultures following brief (5 min) exposure to Glu, most neurons still developed delayed degeneration over the following day[29]. On the other hand, if extracellular Ca^{2+} was removed from the exposure solution, acute neuronal swelling was actually enhanced, although neuronal loss the next day was markedly reduced. If both Na^+ and Ca^{2+} were removed, cortical neurons exposed to Glu showed neither acute swelling nor delayed degeneration. Furthermore, the neurotoxicity of low concentrations of Glu could be enhanced by augmenting extracellular Ca^{2+} (Ref. 29).

These observations suggest that Glu-induced neuronal injury can be separated into two components: an acute, Na^+-dependent component marked by immediate cell swelling, and a delayed, Ca^{2+}-dependent component marked by delayed cell degeneration. Both components, at least *in vitro* where cell swelling is unlimited, are capable of acting in isolation to produce irreversible neuronal injury. However, with lower toxic exposures, the Ca^{2+}-dependent component probably predominates. The delayed nature of this component resembles the neuropathology of ischemic neuronal injury, which can also show a substantial delayed component[30,31].

A similar Ca^{2+} dependence characterizes quinolinate-[32] and homocysteate-induced[33] neuronal injury in cortical cultures, and EAA-induced injury in cerebellar slices[34] and hippocampal cultures[35]. The basis for this dependence of extracellular Ca^{2+} remains to be delineated, although mediation of EAA-induced neurotoxic injury by a Ca^{2+} influx seems most likely, and is supported by three arguments: (1) delayed cortical neuronal disintegration could be mimicked by the Ca^{2+} ionophore A23187 (Ref. 29); (2) isotope flux[36], electrophysiology[26], precipitation[37], and Ca^{2+}-sensitive dye[38–40] studies have established that Ca^{2+} entry accompanies EAA action on central neurons; and (3) the antagonist pharmacology of Glu neurotoxicity, at least on cortical neurons, indicates a disproportionate importance of the *N*-methyl-D-aspartate (NMDA) subtype of Glu receptors, which differ from other Glu receptor subtypes in activating channels that are highly permeable to Ca^{2+} (see below).

How might glutamate induce a toxic Ca^{2+} influx?

Glutamate could potentially produce a toxic Ca^{2+} influx into neurons through four routes, illustrated in Fig. 1. First, Glu could trigger a Ca^{2+} influx directly through chemically gated ion channels, specifically those gated by the NMDA subclass of Glu receptors. Glu acts on both non-NMDA (quisqualate and kainate) as well as NMDA receptors[41], to open membrane channels permeable to Na^+, leading to a Na^+ influx and membrane depolarization[26]. However, only the channels opened by NMDA receptors are, in addition, highly permeable to Ca^{2+} (Ref. 39).

The second route by which Glu could induce a Ca^{2+} influx is through voltage-gated Ca^{2+} channels activated by the Glu-induced membrane depolarization (see R. W. Tsien *et al.*, this issue). Properties of high conductance and slow inactivation make the L subtype likely to contribute a greater net Ca^{2+} influx than T or

N subtypes initially, although L channels appear to depend on cellular metabolism for their functional integrity, and hence may become inactive late in the injury process after metabolic failure occurs. In contrast, T channels do retain function in isolated membrane patches; however, given their small conductance and relatively rapid inactivation, these channels are probably unlikely to make a major contribution to net Ca^{2+} influx. The N subtype may contribute specifically to the Ca^{2+} influx localized to nerve terminals, and thus might be important in enhancing the propagation of injury by triggering further release of endogenous Glu.

The third possible route of Glu-induced Ca^{2+} entry is via the membrane Na^+/Ca^{2+} exchanger, a mechanism that normally acts to extrude Ca^{2+}, but that might operate in reverse under conditions of elevated cytosolic Na^+ (Ref. 42). In addition, energy-dependent Ca^{2+} transporters capable of pumping cytosolic Ca^{2+} out across the cell membrane, or into intracellular compartments (endoplasmic reticulum, possibly mitochondria) are probably impaired under the low-energy conditions produced by lasting Glu action and hypoxia–ischemia (see M. Blaustein, this issue).

The fourth possible route of Ca^{2+} entry is via non-specific membrane leak, perhaps enhanced by the membrane distortion associated with acute Glu-induced excitotoxic swelling. It is of interest to note that enhanced leakage of complexed ^{51}Cr from preloaded neurons – a phenomenon unlikely to occur via membrane channels – can de detected within 15–30 min of onset of toxic Glu exposure[43].

Finally, it should be kept in mind that cytosolic Ca^{2+} concentrations might be elevated not only by an influx of extracellular Ca^{2+}, but also by release from intracellular stores, especially endoplasmic reticulum. Inositol phospholipid metabolism might be activated by direct interaction of quisqualate receptors with GTP-binding proteins[44–46]; this activation could then lead via inositol 1,4,5-trisphosphate production to a mobilization of intracellular Ca^{2+}, a process perhaps itself enhanced by elevated cytosolic Ca^{2+} (see S. Nahorski, this issue) or intracellular acidosis[9].

How does glutamate actually induce a toxic Ca^{2+} influx?

While there are thus multiple possible routes by which Glu action might lead to a Ca^{2+} influx, available data suggest that the NMDA receptor-activated

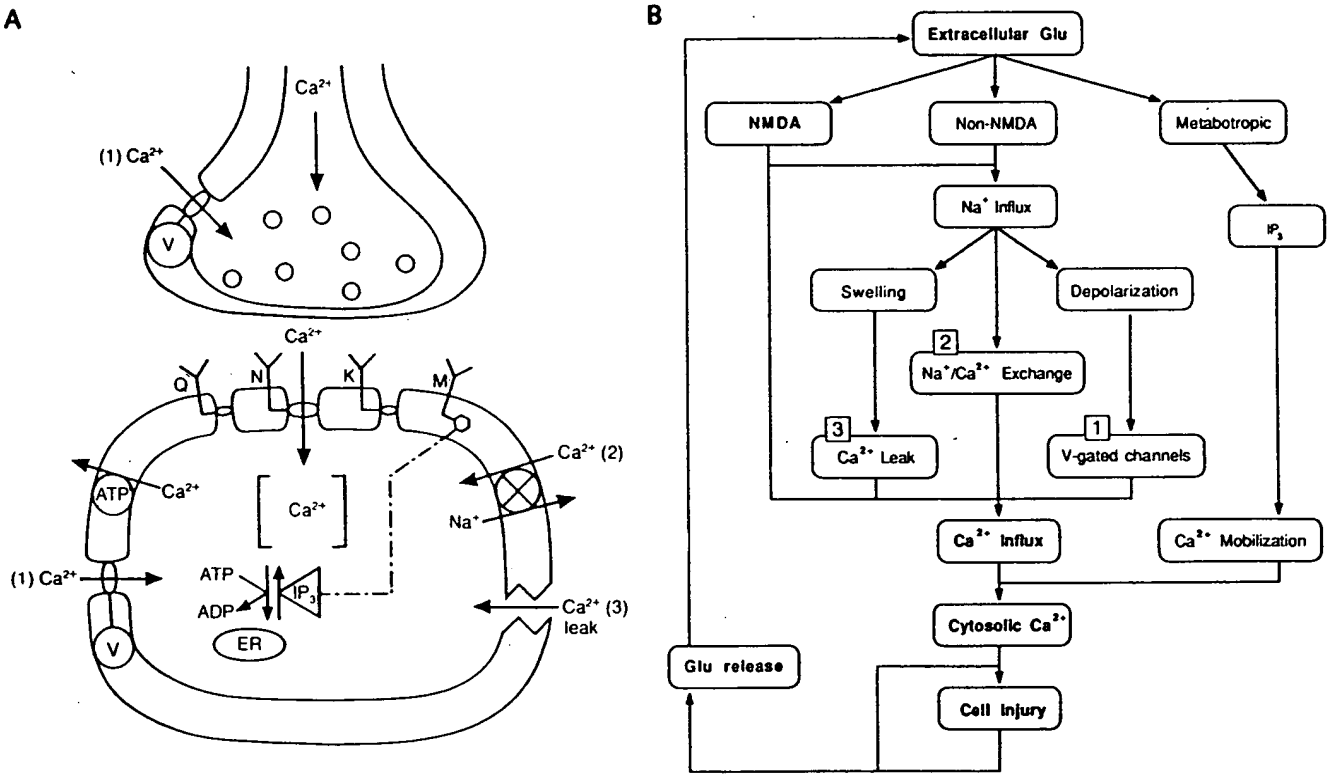

Fig. 1. (A) *Schematic diagram and* **(B)** *flow diagram showing speculative mechanisms linking Glu action to increased neuronal cytosolic Ca^{2+} and resultant cell injury. Glu can lead to Ca^{2+} influx directly through chemically gated channels linked to NMDA (N) receptors (route 1), but not those linked to the quisqualate (Q) or kainate (K) receptors. However, both NMDA and non-NMDA receptors trigger a Na^+ influx, leading to three other routes of Ca^{2+} influx: (route 2) membrane depolarization and activation of voltage (V)-gated Ca^{2+} channels; (route 3) elevated cytosolic Na^+ and reverse operation of the membrane Na^+/Ca^{2+} exchanger; and (route 4) Cl^- and water entry, cell swelling, and transmembrane Ca^{2+} leak. In addition, Glu activation of 'metabotropic'[44–46] (M) receptors, might directly activate inositol 1,4,5-trisphosphate (IP_3) production, leading to the mobilization of Ca^{2+} from endoplasmic reticulum. (Furthermore, under the energy-depletion conditions accompanying hypoxia–ischemia, ATP-dependent pumps normally responsible for transporting cytosolic Ca^{2+} against concentration gradients – either back across the cell membrane or back into endoplasmic reticulum – would be impaired.) Resultant elevated cytosolic Ca^{2+} could lead to neuronal cell injury and more Glu release from neuronal presynaptic terminals. This additional Glu release might, in turn, affect postsynaptic receptors and subsequently propagate injury. This outline is acknowledged to be both speculative and incomplete. In particular, it contains no mention of protein kinase systems (activated by Ca^{2+}, cyclic nucleotides, or diacylglycerol) which could bring about important lasting changes in certain relevant proteins, for example membrane Ca^{2+} channels[67] or enzymes involved in inositol phospholipid metabolism.*

channel may be the predominant route mediating Glu-induced toxic Ca^{2+} influx and neuronal injury. High concentrations of the selective NMDA antagonist, 2-amino-5-phosphonovalerate[41] (APV), as expected, produce only partial reduction in the Glu-induced cortical neuronal excitation and acute swelling, but produce near complete blockade of Glu-induced late neuronal cell loss on most cortical[47] (but see Ref. 48 for an exception) and hippocampal[35] neurons. Blockade of Glu cortical neurotoxicity[47,49] can also be achieved by the non-competitive NMDA antagonists ketamine and dextrorphan[50], and the broad-spectrum antagonist kynurenate[41], but not by the non-NMDA receptor-preferring antagonist γ-aminomethyl sulfonate[41]. These findings suggest that NMDA channel activation is required for Glu-induced neuronal destruction, at least on cortical and hippocampal neurons. Rothman discovered that considerable reduction of neurotoxicity can be achieved even when an NMDA antagonist is added 'late' after conclusion of Glu exposure[35,47]. The protective efficacy of late antagonism may reflect interruption of the continuing propagation of injury by further endogenous Glu release.

It is consistent with these observations that replacement of extracellular Na^+ does not block Glu-induced late cortical neuronal degeneration[29], and mere depolarization (achieved with high K^+ in the presence of Cl^- replacement and a Glu antagonist) does not damage hippocampal neurons[35]. Furthermore, the intracellular Ca^{2+} increase induced by EAAs on single striatal neurons appears to be largely mediated by the NMDA receptor-activated channel[40]. In particular, the Ca^{2+} influx induced by NMDA or Glu is not sensitive to Li^+ (sufficient to block a depolarization-induced Ca^{2+} influx), suggesting a relatively minor contribution from voltage-gated channels to that influx. Nor does replacing extracellular Na^+ with Li^+, an ion probably not recognized by the Na^+/Ca^{2+} exchanger, substantially attenuate the Ca^{2+} influx[40].

This is not to say that activation of non-NMDA receptors is unimportant to Glu neurotoxicity. Co-activation of these receptors may play an ancillary role in NMDA receptor-mediated toxicity, by helping to depolarize cells and thus overcome the voltage-dependent block of NMDA channels by extracellular Mg^{2+} (Ref. 51). In addition, non-NMDA receptors should act in conjunction with NMDA receptors to produce the Na^+ influx and membrane depolarization that underlies both acute excitotoxic neuronal injury, and Ca^{2+} influx via routes other than through the NMDA receptor-activated channel. (Glu-induced Na^+ influx and membrane depolarization would be expected to be necessary for the activation of voltage-gated Ca^{2+} channels, the stimulation of the Na^+/Ca^{2+} exchanger, and neuronal swelling with enhancement of membrane Ca^{2+} leak.) However, a direct implication of assigning central importance to NMDA receptors in mediating Glu neurotoxicity, is that non-NMDA receptors would be expected to be generally less effective than NMDA receptors in inducing neuronal injury. Indeed, with 5 min exposure, both kainate (see above) and the selective quisqualate agonist, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), appear to be far less toxic than NMDA or Glu on most cortical neurons[52].

The observations described above suggest that routes of Ca^{2+} entry other than through NMDA receptor-activated channels are neither sufficient, nor necessary, for most Glu-induced neurotoxic injury. However, present observations do not preclude the possibility that these alternative routes of Ca^{2+} entry (or decreased Ca^{2+} efflux, decreased Ca^{2+} sequestration, or increased Ca^{2+} release from intracellular stores) could play a consequential secondary role under some conditions, acting to amplify the Ca^{2+} influx and resultant injury that can be directly attributed to NMDA receptor-activated channels. Some support for the importance of L channel-mediated influx is provided by experimental suggestions that organic Ca^{2+} channel antagonists may reduce hypoxic–ischemic injury *in vivo*[9,15]. While alterations in cerebral blood flow might account for some of these benefits, there is a recent report that both diltiazem and flunarizine can improve electrophysiological recovery in hippocampal slices exposed to hypoxia[53]. Elucidation of the specific roles of T- or N-type voltage-dependent Ca^{2+} channels in hypoxic injury is likely to depend on the future development of suitable selective pharmacological antagonists.

Implications for the therapy of hypoxia–ischemia

Considerable recent attention has appropriately focused on the strategy of using NMDA antagonists to reduce hypoxic–ischemic neuronal injury[22]. Hypoxic cortical neuronal injury *in vitro* has been found to be systematically amenable to reduction by NMDA antagonists[54], and studies from a rapidly growing number of laboratories have shown beneficial effects of systemically administered NMDA antagonists in animal models of both focal and global brain ischemia[55–66]. Of particular practical importance are promising reports from several investigators that benefit is obtained by administering NMDA antagonists substantially after the initiation of hypoxia–ischemia[56,58,61,66].

However, the general purpose of the Ca^{2+} hypothesis of hypoxic–ischemic injury discussed here is to raise the possibility of additional points of therapeutic attack. If an elevation in cytosolic Ca^{2+} is indeed a critical event in the pathogenesis of hypoxic–ischemic injury, improved therapeutic efficacy might be attained by combining an NMDA antagonist with manoeuvers designed to block other causes of this elevation. Possible targets would include non-NMDA receptors, voltage-gated L or N channels, the Na^+/Ca^{2+} exchanger, or mechanisms governing the sequestration or release of Ca^{2+} from intracellular stores. In addition, this Ca^{2+} hypothesis defines a body of subsequent toxic events – those orchestrated by increased intracellular Ca^{2+} – that could themselves prove amenable to specific remedy.

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Books Received

Review copies of the following books have been received. Books that have been reviewed in full in *TINS* are not included. The appearance of a book in this list does not preclude the possibility of it being reviewed in the future.

Jack D. Barchas and William E. Bunney, Jr (ed.) *Perspectives in Psychopharmacology: A Collection of Papers in Honor of Earl Usdin* Alan R. Liss, 1988. US$120.00 (xxxvii + 681 pages) ISBN 0 8451 2742 X

R. W. Beck and C. H. Smith *Neuro-Ophthalmology: A Problem Oriented Approach* Churchill Livingstone, 1988. £27.00 (ix + 264 pages) ISBN 0 316 08651 7

R. Benecke, B. Conrad and C. D. Marsden (ed.) *Motor Disturbances I* Academic Press, 1987. £37.00 (xi + 333 pages) ISBN 0 12 086840 7

James Paul Dworkin and David E. Hartman *Cases in Neurogenic Communicative Disorders* Churchill Livingstone, 1988. £16.50 (xvii + 397 pages) ISBN 0 316 19751 3

Allen D. Elster *Cranial Magnetic Resonance Imaging* Churchill Livingstone, 1988. £75.00 (423 pages) ISBN 0 443 08542 0

Salvatore Giaquinto *Aging and the Nervous System* John Wiley & Sons, 1988. (viii + 224 pages) ISBN 0 471 91835 0

A. Glass (ed.) *Individual Differences in Hemispheric Specialization (NATO ASI Series A: Life Science* Vol. 1*)* Plenum Publishing, 1987. US$75.00 (ix + 406 pages) ISBN 0 306 42586 6

Douglas R. Gnepp (ed.) *Pathology of the Head and Neck: Contemporary Issues in Surgical Pathology* Churchill Livingstone, 1988. £70.00 (xiv + 680 pages) ISBN 0 443 08495 5

Jeffrey Alan Gray *The Psychology of Fear and Stress (2nd ed.)* Cambridge University Press, 1988. £42.50/ US$22.95 hbk £15.00/US$22.95 pbk (x + 422 pages) ISBN 0 521 27098 7

Exhibit M (10/644,645)

Emerging treatments for stroke in humans

Walter J. Koroshetz and Michael A. Moskowitz

Ischaemic stroke causes loss of brain function in millions of people worldwide each year. Despite the enormity of the problem, no currently approved therapy reduces stroke size or neurological disability. This contrasts with a number of recently developed agents, reviewed here by **Walter Koroshetz and Michael Moskowitz**, which limit infarct size in animal stroke models. Therapies that dissolve clot and restore blood flow, block excitatory neurotransmission, prevent the ischaemic inflammatory response or scavange free radicals have the potential to revolutionize stroke treatment if proven beneficial in ongoing, placebo-controlled clinical trials. Developments in the experimental arena continue to reinforce the need to characterize the pathophysiological stages leading to brain infarction and recovery.

Brain injury resulting from stroke is a major public health problem. In the USA alone, 550,000 strokes occur each year[1]. Stroke afflicts individuals of all ages, but the incidence doubles with each decade over 45 and reaches 1–2% per year in those over 75 years old[1]. Major disability can result with loss of the ability to communicate, ambulate, coordinate or reason. The incidence of stroke has already been reduced markedly by preventive measures aimed at controlling hypertension, hypercholesterolaemia, substance abuse, smoking, and by the use of anticoagulation in specific high risk groups.

Ischaemic stroke commonly occurs due to occlusion of brain blood vessels by blood clot originating from the heart or atherosclerotic arterial plaque. Standard therapy is often ineffective at preventing brain infarction and is meant to support cardiovascular and respiratory function, control intracranial pressure and prevent recurrent stroke. By contrast, a number of experimental agents reduce infarction size in well-controlled animal stroke models and are now being tested in randomized, double-blind trials in patients. Positive results will not only benefit the human condition but will validate those experimental strategies used to develop suitable stroke therapies for humans.

Pathophysiology of brain infarction

New drugs are designed to block specific events in the pathophysiology of ischaemic brain[2]. Many are designed to neutralize the neural processes that make brain tissue exquisitely vulnerable to energy deprivation[3]. The majority of brain ATP is directed towards maintaining and restoring ionic gradients related to synaptic transmission and the action potential. Excitatory neurotransmission comprises much of the brain's energy demand[3] and glutamate is the major excitatory neurotransmitter in brain. In fact, excessive activation of central excitatory amino acid receptors is toxic even in the presence of normal glucose and oxygen (excitotoxicity)[4].

In both brain ischaemia and excitotoxicity, huge increases in intracellular Ca^{2+} activate Ca^{2+}-dependent proteases, kinases, phospholipases and endonucleases, thereby promoting neuronal and glial cell death[4,5]. Free radicals are produced by damaged mitochondria and by the reaction of molecular oxygen with iron released from protein binding sites by proteases, acidoses and oxygenases. Inhibition of many of these biochemical events attenuates infarction in animal stroke models (see Fig. 1).

The therapeutic window

Because most patients receive medical attention minutes to hours after the onset of brain ischaemia, a useful stroke therapy must be efficacious when give hours after stroke onset. Animal experiments demonstrate that only minutes of severe ischaemia or hours of mild ischaemia initiate a sequence of events leading to infarction. However, even in the most severe ischaemic insult the process of tissue destruction may not be completed for hours or even days[6–8]. Understanding the stages of stroke during which a therapy may be effective is critical for drug development.

The peri-infarct area, between the densely ischaemic 'core' and normally perfused brain, contains compromised but salvagable tissue in a zone of reduced blood flow[9,10]. High extracellular levels of excitotoxic

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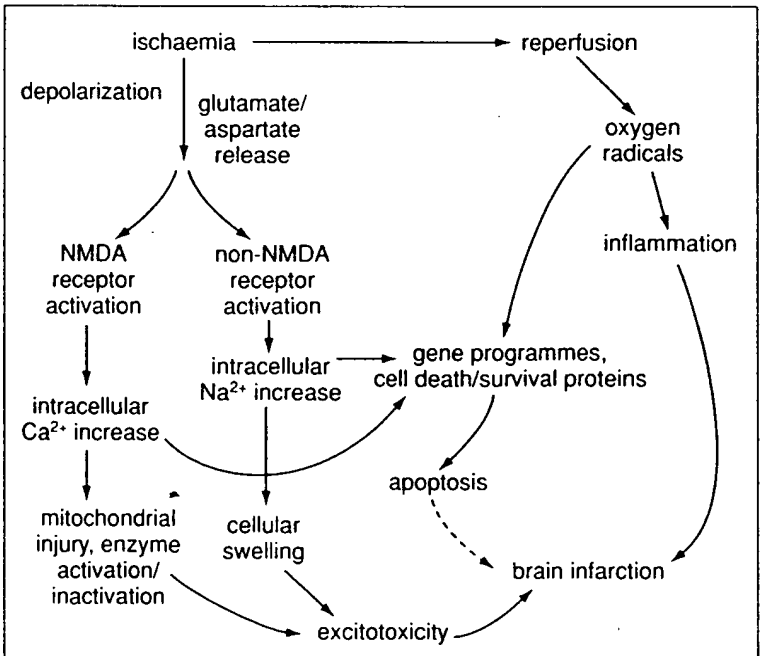

Fig. 1. Sequence of events in stroke.

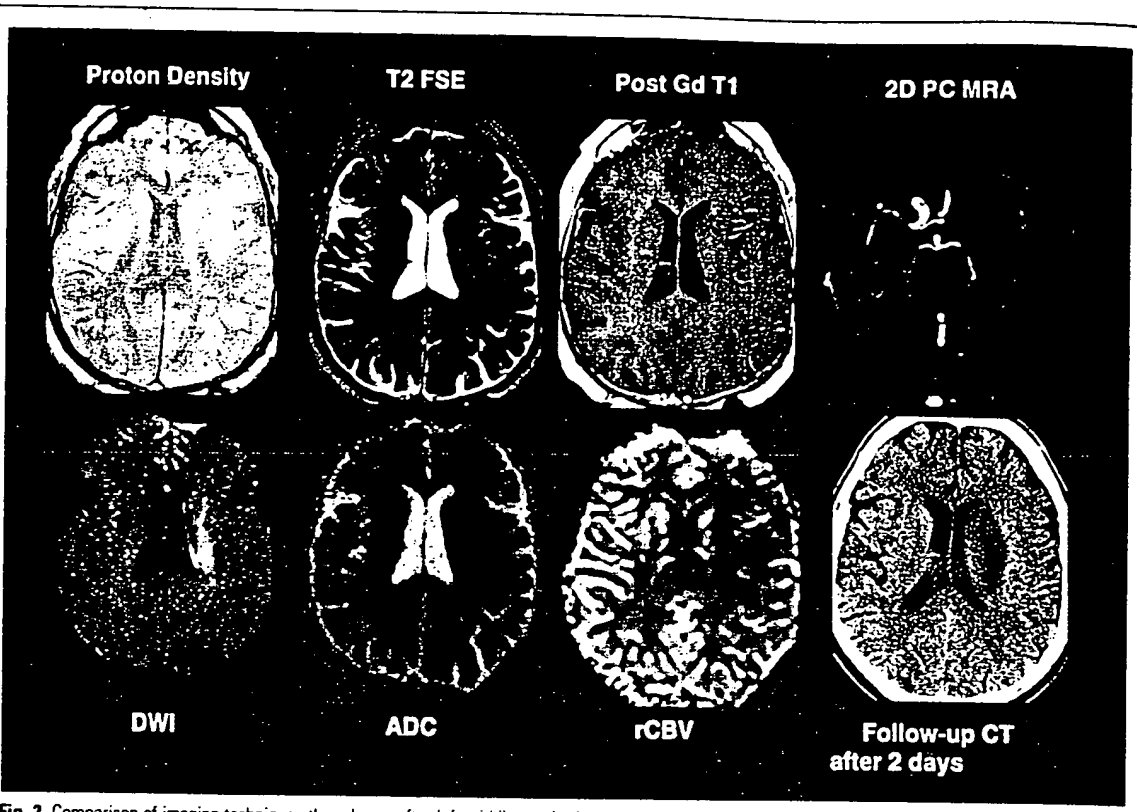

Fig. 2. Comparison of imaging techniques three hours after left middle cerebral artery occlusion. Upper panel: Conventional magnetic resonance images. Lower panel: Regional diffusion weighted image (DWI), apparent diffusion coefficient (ADC) and much larger cerebral blood volume (CBV) abnormalities. (The final stroke was confined to the regions that were abnormal on the initial diffusion scans.)

transmitters[11], waves of cellular depolarization [resembling spreading depression, or anoxic depolarization], free radical production, and inflammation may compromise energy metabolism further. Most neuroprotective agents, e.g. glutamate receptor antagonists[12] or free radical scavengers, attempt to inhibit such 'stressful' activity in the peri-infarct zone and salvage tissue otherwise destined to die in the hours or the day after stroke. Although most investigators agree that eventual tissue recovery is considerably worse without reperfusion, certain events during recirculation (e.g. toxic oxygen radical products and/or reactions produced by invading white blood cells) may negatively affect an otherwise more favourable recovery with restoration of blood flow. Such 'reperfusion injury' may occur in stroke after natural or pharmacological lysis of clot.

The so-called therapeutic window refers to this temporal opportunity for an intervention to prevent ischaemic tissue from progressing to infarction[9,13,14]. Recent positron emission tomography and magnetic resonance imaging studies indicate that the therapeutic window may be much more variable and prolonged in human stroke than in animal models[12,15]. This may relate to the highly variable and heterogeneous levels of ischaemia that occur in human stroke as opposed to the controlled, constant ischaemic insults used in experimental animals. Magnetic resonance imaging may be useful to define the therapeutic window in individual patients by staging the evolution of ischaemic injury as opposed to the more limited indicator, time since onset of symptoms[15]. (See Fig. 2.)

Pharmacological strategies in stroke

Many of the promising experimental neuroprotective agents target specific neural properties thought to underlie the brain's unique susceptibility to ischaemic injury. Others are more general and potentially useful in protecting a variety of tissues from infarction. Each has been shown to be partially beneficial in limited, well-controlled animal stroke models but, with the possible exception of tissue plasminogen activator, not definitively in human stroke.

Table 1 lists drugs that are representative of the treatment strategies or classes that appear most promising. Some of these drugs have also shown promise when tested in animal models of traumatic brain injury or in head-injured patients [e.g. superoxide dismutase linked to polyethylene glycol (PEG-SOD), tirilazad]. Successful brain protection in head injury may not necessarily predict success in stroke patients and vice versa. However, ischaemia and reperfusion develops commonly in both conditions. Importantly, drug-induced behavioural effects often limit the doses used in current stroke trials. Higher, and potentially more efficacious, doses of brain protective drugs can be ethically administered to comatose-head injured patients.

Block of glutamate receptor actions

NMDA receptor antagonists or channel blockers reduce the size of focal ischaemia in many animal models

Table 1. Clinical trials in acute stroke

Drug	Action	Company	Phase
Block of glutamate receptor actions			
Cerestat	channel blocker	Boehringer-Ingelheim	III, trauma
Dextrorphan	channel blocker	Hoffman-LaRoche	I
Dizocilpine	channel blocker	Merck	I
Remacemide	channel blocker	Astra	I
Eliprodil	NMDA receptor antagonist	SyntheLabo Lorex	III
Selfotel	NMDA receptor antagonist	Ciba-Geigy	III
YM90K	AMPA receptor antagonist	Yamanouchi	I
Free radical scavengers			
Tirilazad	Fe^{3+} generated free radicals, at vascular wall?	Upjohn	III, trauma/SAH
Pergorgotein	PEG-superoxide dismutase	Sterling-Winthrop	II, trauma
Anticoagulation and thrombolysis			
Prourokinase	intra-arterial fibrinolytic	Abbott	II
Arvin	intravenous defibrinogenation	Knoll	III
Alteplase	intravenous fibrinolytic	Boehringer-Ingelheim, Genentech	III
Orgaron	heparinoid anticoagulant	Organon	III
Nadroparin	heparinoid anticoagulant	Sanofi	III
Streptokinase	intravenous fibrinolytic	Pharmacia	III
Anti-inflammatory agents			
Enlimomab	wbc adhesion blocker	Boehringer-Ingelheim	III
Anti CD-11 antibody	wbc adhesion blocker	Repligen	I
Voltage-gated channel blockers			
Lubeluzole	Na^+ channel blocker	Jansen	III
	Na^+ channel blocker	Glaxo-Wellcome	III
Riluzole	Na^+ channel blocker	Rhône-Poulenc Rorer	I
Fosphenytoin	Na^+ channel blocker	Parke-Davis	III
SNX111	Ca^{2+} channel blocker	Neurex	I
Nimodipine	Ca^{2+} channel blocker	Syntex	III
Lifarizine	Ca^{2+} channel modulator	Syntex	II
Neurotrophins			
Trofermin	binds to bFGF receptors	Scios Nova	II
Potentiation of inhibitory neurotransmission			
Chlormethiazole	potentiates GABA	Astra	III
Stroke recovery			
Citicholine	phospholipid precursor	Interneurone	III

PEG, polyethylene glycol; SAH, subarachnoid haemorrhage; wbc, white blood cell

but are less effective in models of global ischaemia (e.g. after cardiac arrest)[9]. Under conditions of high glutamate concentration when the channel is more likely to be open, drugs that block the NMDA receptor channel have a theoretical advantage over drugs that compete with glutamate for receptor binding. Glycine site (e.g. MDL105572, AEE1021, L687414) or potential polyamine modulatory site (eliprodil) antagonists may be neuroprotective because the binding of glycine and polyamines to separate allosteric sites promotes NMDA receptor channel opening. In preclinical studies, putative glycine or polyamine site antagonists reduced infarct size and possessed significantly fewer behavioural effects than NMDA receptor antagonists in experimental animals.

The prototype channel blocker dizocilpine[16] was targeted for stroke trials in the mid 1980s. Development was halted, because normal animals developed vacuolar changes within cingulate gyrus neurones[17]. This has also occurred with high doses of other NMDA receptor antagonists. NMDA receptor antagonists cause pronounced psychomimetic effects, which may directly or indirectly relate to antagonism of specific NMDA receptor subtypes. If the drugs indeed improve the final clinical outcome, most behavioural effects (which are transient and vary among different compounds) would be considered tolerable as an alternative to the permanent deficits of stroke.

Non-NMDA receptor (e.g. AMPA receptor) activation can produce significant ionic flux and excitotoxicity during ischaemia because, unlike NMDA receptors, they are not inhibited by acidic pH in the ischaemic zone. Non-NMDA channel blockers [e.g. NBQX; 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline] and other quinoxaline-dione derivatives provide neuroprotection in animal models of focal and global ischaemia. The therapeutic window is reportedly longer for NBQX (which also has antagonist properties at the glycine site of the NMDA

Box 1. Cellular mechanisms of ischaemic brain injury

Under conditions of normal metabolism, glutamate receptor activation causes cations to flow into neurones through membrane channels; subsequent membrane depolarization mediates action potentials, elevation of intraneuronal Ca^{2+} concentrations and further neurotransmitter release. Return to the resting membrane potential requires energy-dependent cation transport.

As blood flow and ATP levels fall in stroke, there is an 'orderly' disruption of cellular processes within both grey and white matter. With moderate decreases in perfusion, protein synthesis decreases[1]. Subsequently, as ATP levels fall, neuronal function and electrical activity cease[2]. Finally, cation pumps fail and massive neuronal depolarization occurs. Potassium passes from the cell to the shrunken extracellular space and contributes to widespread depolarization[3]. Leakage of glutamate from the synaptic cleft and intracellular stores increase extracellular levels 25–100 times higher than normal[4]. Depolarization also opens voltage-gated Na^+ and Ca^{2+} channels. Huge rises in intracellular Ca^{2+} occur due to the action of glutamate. This stimulates widespread neurotransmitter release, thereby activating a host of neurotransmitter receptors and their second messenger systems, including NO synthase, calpain, phospholipase A_2 (Ref. 5), a variety of kinases, and endonucleases. Gene programmes, some protective and some destructive, are activated. Attempts are made to sequester the raised Ca^{2+} in mitochondria. High mitochondrial Ca^{2+} may actually damage mitochondrial function and lead to free radical production upon the return of oxygen to mitochondrial oxidases[6]. During ischaemia, brain metabolism proceeds anaerobically and lactic acid is generated[7]; tissue pH drops below 6.8 (Ref. 8). If blood flow is restored, oxygen may participate in biochemical reactions that generate toxic free radicals at the endothelium, inside the brain cells and possibly in the extracellular space[9,10]. White blood cells adhere to the wall of the endothelium and gain entry into the ischaemic brain tissue within 24 hours.

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receptor), than with selective NMDA receptor or channel antagonists. NBQX is also effective in severe ischaemic states (e.g. global ischaemia)[18,19] in which NMDA receptor antagonists are less neuroprotective. The AMPA receptor antagonist, YM90K, is in phase I trials.

Free radical scavengers

Oxygen free radicals are highly reactive species that promote damage to lipid, DNA, carbohydrates and protein and contribute to the process of excitoxic neuronal death (see Fig. 1). Free radical production contributes to the breakdown of the blood–brain barrier and brain oedema, movement of white cells into the ischaemic zone and alterations in blood flow[20,21]. Levels of endogenous free radical scavenging enzymes and oxidized glutathione fall during ischaemia whereas free radical production increases during reperfusion. NO is a weak free radical that can combine with superoxide to form a more toxic species (e.g. reactant products of peroxynitrite anion).

Free radical scavengers and/or inhibitors of lipid peroxidation reduce brain damage after ischaemia, particularly during reperfusion. In fact, mice overexpressing SOD are more resistant to neuronal death after transient cerebral ischaemia associated with reperfusion injury[22]. U74006F is a 21-amino acid steroid that reduces stroke damage in models of transient ischaemia (ischaemia/reperfusion stroke models)[23,24]. The drug was almost simultaneously evaluated for efficacy in three major clinical trials: head injury, stroke, and ischaemic brain injury due to vasospasm after subarachnoid haemorrhage. In the vasospasm trial, there was evidence that patient outcome was improved in males treated with tirilazad; women were found to metabolize the drug more rapidly. A higher dose trial in women is currently under way. The trial in ischaemic stroke was suspended because the preliminary data (600 patients) suggested that the trial had a high likelihood of not showing net clinical improvement. A stroke trial of higher dose U74006F is currently under way.

PEG-SOD reduces stroke size in animal models in the presence of reperfusion[25,26]. PEG-SOD improved the outcome of severe head injury in 104 patients treated within four hours of trauma in a randomized multi-centred controlled phase II trial[27]. After three months, 44% of patients in the placebo group were in a vegetative state or died as compared to 20% of patients in the group receiving PEG-SOD; these differences were sustained for at least an additional three months. 'Spin traps' and other free radical scavengers that readily penetrate into the brain might impart even greater brain protection than PEG-SOD or tirilizad, which work primarily within the vasculature. Since free radical scavengers are most effective in animal models of transient ischaemia their benefit in humans may not be realized until paired with an agent that causes reperfusion (i.e. a thrombolytic).

Hypothermia

Hypothermia commonly provides neuroprotection during neurosurgery but may also protect even when

initiated after the onset of ischaemia. Hypothermia remains the single most effective strategy for reducing ischaemic damage[28]. Brain temperature of 33–34°C is sufficient to protect in animal experiments although duration may be critical as worsening has been noted after prolonged (>8 hours) hypothermia[29]. Brain cooling reduces brain oxygen consumption and metabolism and delays depletion of cytoplasmic ATP stores. Post-ischaemic hypothermia reportedly attenuates primary and delayed excitotoxic processes, breakdown of the blood–brain barrier and the generation of free radicals and lipid peroxidation. Achieving subnormal brain temperature is technically difficult in the acute stroke patient but it is a strategy that deserves clinical evaluation.

Thrombolysis and anticoagulation

Improved outcome was recently reported from Hong Kong in a stroke trial of nadroparin, a low molecular weight heparin-type anticoagulant[30]. Anticoagulation with heparin has long been used by some stroke neurologists in selected patients.

Urokinase, streptokinase, recombinant tissue plasminogen activator (rt-PA), and prourokinase are drugs that dissolve clots by selective cleavage of fibrin matrix through the activation of plasmin. Thrombolytics benefit patients with heart attack if administered early. In the heart they promote vessel recanalization (70–80%) but carry an increased risk (0.6%) of fatal brain haemorrhage. Several groups[31] have treated patients with intra-arterial urokinase, dissolving clot in the basilar artery (an almost certainly fatal condition) and have observed rapid and significant reversal of deficits. The hazard of thrombolysis relates to fatal haemorrhage into infarcted brain regions. This complication, combined with lower recanalization rates after intravenous administration, resulted in multiple negative trials of intravenous streptokinase and urokinase in stroke.

Considerable interest has arisen from recently published results of stroke trials evaluating intravenous rt-PA. In a European trial, patients were treated within six hours of stroke onset and there was a higher rate of intracranial haemorrhage and mortality in the treated group. However, clinical benefit of rt-PA was suggested by improved outcome in the subgroup enrolled without ischaemic changes on CT scan[32]. Most importantly, the NINDS study demonstrated clear benefit in the treated group[33]. Half of the patients enrolled were treated within 90 minutes of stroke onset and none were treated after three hours. The haemorrhage rate was increased from 0.6% to 6.4%, but overall mortality was not increased by treatment. This positive trial may have broad implications for stroke therapy in patients presenting within two to three hours of their stroke onset and may impact on the design of future clinical trials. It should also encourage the development of agents that decrease the risk of reperfusion injury with haemorrhage after thrombolytic therapy.

Anti-inflammatory agents

Within hours of stroke, white cells adhere to the blood vessels in the ischaemic zone[34]. This inflammatory reaction may occlude capillaries and obstruct regional blood flow even during reperfusion. White cells traverse the blood brain barrier 12–48 hours after onset[35] and may provide a rich source of oxygen free radicals. Eventually the infarcted zone is infiltrated with lymphocytes, polymorphonuclear cells and macrophages. Cytokines, destructive chemicals from PMNs and macrophages trapped in the microcirculation or invading the tissue may contribute to vessel wall injury, haemorrhage, oedema and tissue necrosis. Ischaemic injury is reduced by agents that cause neutropaenia[36] or limit white cell adhesion to endothelial receptors, such as antibodies to ICAM and P-selectin[37,38]. A clinical trial of anti-ICAM monoclonal antibodies in stroke is currently under way.

Voltage-dependent channel blockers

Neurotransmitter release is triggered by opening Ca^{2+} channels in the axon terminal depolarized by Na^+ channel activation. Only L channel blockers have been used clinically (nimodipine) and they do not block the Ca^{2+} channels necessary for central neurotransmitter release. They may, however, prevent excessive Ca^{2+} influx into cytoplasmic and mitochondrial compartments and Ca^{2+} influx appears important to ischaemic injury in brain white matter[39].

L channel blockers were studied in multiple large clinical trials but were without obvious beneficial effects on overall outcome. There is a suggestion from retrospective meta-analysis that patients with moderate-severe initial deficits treated within 12 hours had a more favourable clinical outcome than those randomized to placebo[40]. A follow-up clinical trial in The Netherlands found that the nimodipine-treated group actually had a worse outcome. Nimodipine improves outcome in patients at risk for stroke due to vasospasm after subarachnoid haemorrhage[41].

A variety of N channel blocking peptides from spider venom and marine animals block neurotransmision effectively. Drugs that reversibly mimic these actions may prove important neuroprotective agents[42]. SNX111 is the compound in the most advanced clinical development, although significant hypotension arising from N channel-mediated sympathetic block may pose unacceptable risks in stroke patients.

Lamotrigine, lubeluzole, riluzole and fosphenytoin (the pro-drug for phenytoin) reduce ion flux through Na^+ channels and attenuate the release of neurotransmitter from axon terminals. Many are known to be safe and effective for treating epilepsy (e.g. phenytoin and lamotrigine), another condition in which there is excess excitatory transmission, and have been reported to decrease ischaemic injury in animal stroke models. Riluzole reportedly slows the course of bulbar amyotrophic lateral sclerosis, but its mechanism of action remains unclear. Enrollment in a clinical trial of lubeluzole has been completed and results are pending.

New strategies on the horizon

NO

A strategy that enhances vascular and limits neuronal NO synthase is promising in stroke[43]. Endothelial NO causes vasodilatation, increases cerebral blood flow and limits infarct size in animal models. However, excessive parenchymal neuronal NO synthesis promotes cell death, possibly via reactive metabolites related to peroxynitrite anion formation, activation of ADP-ribosylation, binding to iron–sulphur complexes. NO appears to be an important mediator of excitotoxic injury in stroke[44,45]. Mice deficient in neuronal isoform of NO synthase develop smaller infarcts following middle cerebral artery occlusion[46]. Compounds that specifically inhibit neuronal NO production (e.g. 7-nitroindazole), but do not block vasodilatation due to endothelial NO production, appear promising in experimental ischaemia[47].

Neurotrophins

A variety of neurotrophins or growth factors limit stroke size in animal models. For example, both the intraventricular and intravenous administration of fibroblast growth factor (FGF) decrease infarct size when administered acutely[48,49]. In addition to a direct neuroprotective effect, fibroblast growth factor dilates blood vessels through mechanisms involving NO release from the endothelium. FGF synthesis in the brain begins days after ischaemia and may participate in restorative processes occurring days to weeks later. The action of neurotrophins may be mediated through activation of protective genetic programmes (see below). A trial in stroke patients was recently initiated.

Altered gene expression

Recent studies have also shown that brain ischaemia promotes transcription of gene programmes, which can both positively and negatively impact stroke outcome. Heat shock proteins[50,51] and protein synthesis triggered by growth factors protect cells[52]. Genes, such as *Bcl-2* (Ref. 53) which block 'death genes' have been identified[54]; in addition they block cell death in a variety of paradigms previously considered representative of necrotic cell death. Genes that cause apoptotic cell death may be expressed in ischaemic brain[55]. Such powerful, endogenous, genes and gene products may have a significant effect on future ischaemic therapies for brain and other tissues.

Additional therapeutic strategies

Drugs that inhibit the consequences of high intracellular Ca^{2+} (e.g. cell permeable, Ca^{2+} chelators), adenosine analogues that reduce transmitter release, inhibitors of Ca^{2+}-activated proteases (such as calpain), thrombin, endonucleases (aurintricarboxylic acid) and phospholipases; κ-opioid receptor antagonists, GABA receptor agonists (chloromethiazole is in clinical trials), as well as antisense knockout of destructive proteins may develop into clinically significant treatments. Partial or even complete recovery of neurological function after stroke occurs commonly, especially in young stroke victims. Research focused on how function is redistributed in brain after focal injury may lead to therapies that enhance recovery; CDP-choline, which may promote new membrane synthesis, is currently being tested for its ability to promote functional recovery after stroke.

Establishing efficacy in human stroke

Despite the ability of many agents to attenuate stroke size in experimental models, a number of major obstacles impede the development of promising drugs for clinical application.

Improvement in functional level

Protective agents are developed to decrease stroke size. However, besides size of infarction, a variety of other factors, especially patient age and the brain location of the infarct, influence the eventual outcome as measured by functional rating scales. Objective measures are needed to determine whether investigational drugs actually produce their intended therapeutic effect at the doses used in humans. There is a need for measures that predict the extent of at-risk tissue at an early stage.

MR spectroscopy may provide this information[15,56]. MR spectroscopy can reveal the distribution of lactate, and provide information about the status of energy metabolites, neurotransmitters and lipids. MR angiography identifies the major vessels of interest and can localize the site of occlusion. Haemodynamic MRI (HMRI) detects and localizes blood volume and flow abnormalities. Energy failure very early in stroke causes movement of water into the more ordered intracellular compartment. This can be detected as lowered diffusability by diffusion-weighted MRI (DWMRI)[57]. Patient MRI studies suggest that combined HMRI and DWMRI acutely identifies the entire territory at risk with a portion of the ischaemic penumbra defined by the volume of underperfused brain tissue (HMRI) in which the DWMRI and T_2 intensity are still normal[15]. This technology may make it possible to compare a brain's image obtained prior to treatment with an image of the final stroke and then determine whether at-risk brain tissue was preserved by a therapeutic intervention[15].

Clinical costs

The cost of clinical testing is prohibitive. At present, hundreds of patients (600–2000) are required to demonstrate statistical benefit. Reliable surrogate outcome measures would decrease the need for these large numbers.

Combination therapy and sequential treatments

A combination of both neuroprotection (e.g. hypothermia, glutamate receptor antagonists, Na^+ or Ca^{2+} channel blockers or free radical scavengers to prevent reperfusion injury) with efforts to re-establish normal blood flow (thrombolysis) may expand the time window in stroke

treatment. Although agents are now tested individually, once the temporal evolution of ischaemia is better characterized in humans, there will be a need to administer therapeutic drugs in combination.

Trial regimens

In clinical trials, the duration of treatment is arbitrarily set without regard to the individual's pathophysiologic state. Hopefully new MRI techniques will allow staging of brain ischaemia in patients as noted above.

Time frame for onset of treatment

Many patients do not come to medical attention until many hours after stroke onset. Moreover, the number of centres that can accommodate one or more clinical stroke trials is presently limited. The Brain Attack Coalition, the American Heart Association and the National Stroke Association in the USA are attempting to increase public and professional awareness of the need for acute stroke care.

Predictability of animal models

Animal stroke models mimic only a subset of human syndromes, and species differences may be important. Moreover, aged animals are rarely used for experimentation and atherosclerosis is not a confounding factor as it is in the at-risk patient population.

Complexity of stroke syndromes

Human clinical stroke syndromes vary widely in their pathogenesis, clinical expression, course, outcome and likelihood of response to a specific neuroprotective agent. Most clinical stroke trials assess all stroke types together, which may decrease the chance of determining efficacy.

Future goals

Acute stroke intervention is entering its infancy. Objectives for the future include efforts to: (1) refine animal models to better reflect human stroke subtypes; (2) discover agents that protect against specific pathophysiologic events; (3) combine agents with protective properties that are additive; (4) determine the time frame during which an intervention is likely to be effective; (5) continuously monitor the conditions in ischaemic brain that predict the effectiveness of a particular strategy; and (6) educate the public regarding the need for emergency evaluation. Time, effort, resources and the courageous participation of stroke victims will ensure the development of effective stroke therapy in the future.

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Models of neuronal injury in AIDS: another role for the NMDA receptor?

Exhibit N (10/644,645)

Stuart A. Lipton

As many as two-thirds of patients with acquired immunodeficiency syndrome (AIDS) eventually suffer from neurological manifestations, including dysfunction of cognition, movement and sensation. How can human immunodeficiency virus type 1 (HIV-1) result in neuronal damage if neurons themselves are not infected by the virus? In this article Stuart Lipton reviews a series of experiments from several different laboratories that offer related hypotheses accounting for neurotoxicity in the brains of AIDS patients. There is growing support for the existence of HIV- or immune-related toxins that directly or indirectly lead to the injury or demise of neurons via a potentially complex web of interactions between macrophages (or microglia), astrocytes and neurons. However, a final common pathway for neuronal susceptibility appears to be operative, similar to that observed after stroke, trauma and epilepsy. This mechanism involves voltage-dependent Ca^{2+} channels and NMDA receptor-operated channels, and therefore offers hope for future pharmacological intervention.

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AIDS can result in multiple neurological symptoms including deficits in cognitive function and motor sequencing (formerly called the AIDS dementia complex), myelopathy, peripheral neuropathy, and visual dysfunction[1–4]. A consensus panel recently suggested that the syndromes affecting the CNS could be grouped under the rubric of 'HIV-1-associated cognitive/motor complex'[5]. These abnormalities can occur not only in the absence of superinfection by opportunistic organisms, but also in the absence of direct infection of neurons by HIV-1. Originally, only white matter or glial lesions were thought to underlie nervous system dysfunction[4,6,7], but recent morphometric evidence has shown that a substantial degree of neuronal loss can also occur in both the cortex and the retina[8–12]. Axonal loss has been found in the optic nerve, and indicates that retinal ganglion cells have been injured[12]. In the frontal cortex, the loss of neurons has been reported to be between 18 and 38% (Refs 8, 10). In a recent study by Wiley and colleagues[9], cell counts of large neurons (200–500 μm^2) in three areas of neocortex (mid-frontal, inferior-parietal, and superior temporal) were decreased by 30–50% compared to those in controls. Furthermore, confocal laser imaging of the neuropil in brain tisue with HIV encephalitis revealed abnormal dendritic processes, often vacuolated and sometimes sparsely branched and tortuous, in addition to synaptic loss[9]. Moreover, the destruction of white matter may partially reflect loss of axons and therefore another form of neuronal injury. To date, there has been no cytopathological evidence of acute excitotoxic cell death in the brains of AIDS patients, although a chronic, on-going insult remains possible. In fact, the evidence that treatment of early dementia may in part reverse its course might be an indication that remediable neuronal injury precedes irreversible nerve cell loss. Currently, HIV- or immune-related toxic factors are thought to account, at least in part, for the newly recognized neuronal damage.

Neurotoxic properties of HIV-1 peptides or HIV-infected monocytoid cells

One neurotoxin is the HIV-1 envelope glycoprotein gp120 or a fragment of this molecule (Fig. 1)[13,14]. This coat protein has a molecular mass of 120 kDa (hence, the name) and is shed by the virus; gp120 fragments could quite possibly be released from HIV-infected monocytoid cells (monocytes, macrophages or microglia) that harbor the virus in the CNS[15–19]. In tissue culture experiments performed on mixed rodent cultures containing neurons, astrocytes and uninfected monocytoid cells, low picomolar concentrations of gp120 produce an early rise in neuronal intracellular Ca^{2+} concentration[14] and, subsequently, neuronal injury[13,14]. This form of toxicity is in some ways reminiscent of that mediated by excessive stimulation of the NMDA subtype of excitatory amino acid (EAA) receptors (reviewed in Refs 20–22). Such increases in intracellular Ca^{2+} are thought to represent a final common pathway for a diverse group of acute and chronic neurological insults that induce neuronal cell death, ranging from stroke to neurodegenerative dementias[20–22]. The effects of gp120 are dose dependent, with picomolar levels causing increases in intracellular Ca^{2+} and subsequent neuronal injury. In contrast, nanomolar concentrations of gp120 (10–1000 times more concentrated than those solutions used in the experiments just mentioned) have been reported not to be toxic to neurons[13,23] and may block NMDA receptor-operated ion channels, actually preventing NMDA-elicited increases in Ca^{2+} influx[23]. Thus, the dose–response curve of gp120-induced neuronal injury has an inverted 'U' shape[13].

Additional evidence that low concentrations of gp120 are associated with neuronal injury has come from preliminary experiments performed *in vivo*. Brenneman, Hill and co-workers have found that intraventricular injections of picomole quantities of gp120 into rats result in dystrophic neurites in hippocampal pyramidal cells as well as behavioral deficits[24–26]. This evidence also points to a potential role of gp120 in a neurodegenerative process. However, it is as yet unknown if the adverse effects of gp120 act directly on neurons, or act via glial cells such as astrocytes, or by a combination of mechanisms (Fig. 1).

Recently, the HIV-1 nuclear protein *tat* was also shown to be neurotoxic to glioma and neuroblastoma cell lines *in vitro* and to mice *in vivo*[27]. The basic region of the peptide (amino acid residues 49–57) apparently acts nonspecifically to increase the leakage conductance of the membrane, thus altering cell permeability. Further work is necessary to attempt to relate these findings to the neuro-

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pathology encountered in the brains of patients with HIV-1-associated cognitive/motor complex.

Another line of experimentation performed independently by Giulian and colleagues[18] and by Pulliam and co-workers[19] involves HIV-infected monocytes or macrophages. These infected monocytoid cells secrete an as yet unidentified toxic factor(s) that kills rodent, chick and human neurons *in vitro* (Fig. 1). It is not yet known if this macrophage toxic factor(s) might be a gp120 fragment or, alternatively, if gp120, in the absence of HIV-1 infection, might be capable of activating macrophages to release such a toxic factor(s). The latter hypothesis is tenable because the major route of HIV-1 entry into macrophages is mediated by gp120 binding to the surface protein CD4; it is possible that the interaction of gp120 with CD4 or another surface receptor is sufficient to activate macrophages.

Pharmacology of protection from HIV-related neurotoxicity

In some neuronal cell types, including rodent retinal ganglion cells in culture, gp120-induced neurotoxicity can be prevented by (1) antagonists of the L-type voltage-dependent Ca^{2+} channel, such as nimodipine and nifedipine[14], or (2) antagonists of the NMDA receptor–channel complex, such as D-2-amino-5-phosphonovalerate (APV) and dizocilpine (MK-801)[28,29]. Other Ca^{2+} channel antagonists, such as flunarizine and possibly verapamil, may also be able to attenuate the neuronal injury associated with exposure to gp120 (Ref. 30). (The action of flunarizine may not be totally specific for L-type channels; therefore, inhibition of other Ca^{2+} channel types cannot be excluded.) The findings with specific antagonists suggest that activation of both voltage-dependent Ca^{2+} channels and NMDA receptor-operated channels are necessary for this form of HIV-related neurotoxicity, but neither channel type alone is sufficient for neuronal injury. The reason for this dual requirement may involve an important link between NMDA receptor-operated channels and voltage-activated Ca^{2+} channels: both are permeable to Ca^{2+} (Refs 31–33); therefore, both channel types could contribute to an excessive influx of Ca^{2+} associated with subsequent neuronal injury[34–41]. The relative contribution of each channel type to this pathological process may depend on its relative abundance in the plasma membrane of a given neuronal cell type[42]. In fact, since EAA glutamate-like agonists both (1) directly activate NMDA receptor-operated channels, and (2) depolarize neurons to stimulate indirectly voltage-dependent Ca^{2+} channels, then the gating of the two channel types is often modulated in parallel. Along these lines, *either* Ca^{2+} channel antagonists *or* NMDA antagonists can prevent NMDA receptor-mediated neurotoxicity produced in cultured neocortical, hippocampal and retinal ganglion cell neurons by unidentified endogenous EAAs or quinolinate (see below)[39–41].

Another possible link between the effects of gp120 and NMDA-receptor activation arises from the observation that one form of neuronal injury in both the brains of AIDS patients[9] and the brains of rats[24–26] injected with gp120 involves dystrophic neurites. These neurites are excessively tortuous and display a paucity of branches. Some of these neurites may be retracting, giving them a 'bald' appearance. A similar pattern of dystrophic neurites, including retraction of growth cones, has been found in response to sublethal concentrations of NMDA or glutamate in cultured rat retinal ganglion cells and hippocampal neurons[43–45]. Furthermore, these effects are dependent on the influx of Ca^{2+} into the neurons. These findings indicate that the endpoints used to measure neuronal injury related to gp120 or excitotoxicity should include more subtle changes than simply death of neurons, and these alterations in neuronal cytoarchitecture could have important consequences for normal neuronal function and plasticity[45].

Taken together, the simplest explanation for gp120-induced increases in the intracellular concentration of Ca^{2+} and subsequent neuronal injury is that they are mediated by activation of the NMDA receptor. However, the next series of experiments that was performed suggests that the relationship is more complicated than this. First, in patch-clamp recordings, gp120 itself does not appear to be a glutamate-like agonist of NMDA receptors or to increase NMDA-elicited electrical responses in retinal ganglion cells[29]. Secondly, and quite surprisingly, degradation of the endogenous EAA glutamate (which is present in the cultures at ~25 μM, and which by itself is not toxic to retinal ganglion cells) also protects these neurons from gp120-induced injury[29]. This result suggests that *both* gp120 and glutamate-related molecules are necessary for neuronal cell damage; they act synergistically because neither one alone is sufficient to produce neurotoxicity at the low concentrations tested. One possibility is that gp120 directly or indirectly sensitizes neurons to the lethal effects of EAAs acting at the NMDA receptor. However, it remains to be elucidated if gp120 is acting (1) downstream from the neuronal membrane, for example, to activate second messengers such as phosphoinositides and protein kinase C[46–48], or (2) upstream from the neuronal membrane, for example, releasing a toxin from macrophages or microglia[18,19].

The hypothesis that the sensitivity to EAAs might be enhanced by gp120 is particularly intriguing since Heyes *et al.*[49,50] have found that quinolinate, a weak NMDA agonist, is increased in the cerebrospinal fluid of AIDS patients, and the levels of this substance are positively correlated with the degree of dementia. If the experiments in tissue culture with gp120 hold true in the intact brain, then neurons in the brains of AIDs patients are not only exposed to high concentrations of quinolinate but may in addition be unusually vulnerable to them.

One provocative possibility is that gp120 activates macrophages to secrete quinolinate. This would occur, for example, during the process of macrophage infection with HIV-1, when gp120 interacts with the surface receptor CD4. The combination of quinolinate and other endogenous EAAs such as glutamate could potently stimulate NMDA

Direct neuronal injury. One possibility, although not the most likely, is that neuronal injury is directly mediated by gp120 or by a fragment of gp120 that is shed by the virus[13]. According to this hypothesis, gp120 or its fragment would be released by infected macrophages or microglia. Specific NMDA antagonists or degradation of endogenous glutamate prevents gp120 neurotoxicity[29]. Thus, it appears that concurrent binding of excitatory amino acids (EAAs) at the NMDA receptor is also necessary for gp120-induced neuronal injury.

Astrocyte-mediated neuronal injury. Another possibility is that gp120 or a gp120 fragment, possibly from infected macrophages or microglia, binds to astrocytes. The gp120 glycoprotein or its fragment might compete with vasoactive intestinal polypeptide (VIP), or possibly peptide T, for an as yet unidentified receptor that might be related to CD4, but that might equally well be another type of receptor[13,59–62]. In theory, this same competition could occur for neuronal receptors as well as for astrocytic receptors. Normally, VIP may influence the release of neuronal growth factors by astrocytes, such as protease nexin I[55]; it might be postulated that gp120 interferes with this and similar VIP-mediated processes (dotted lines, each marked with an *X*).

Macrophage-mediated neuronal injury. Strong evidence now exists that human monocytoid cells, such as macrophages or microglia, infected with HIV-1 secrete toxic factors *in vitro* that lead to neuronal destruction[18,19]. It is also possible (but not yet proved) that purified gp120 might activate macrophages to secrete such factors in the absence of HIV-1 infection. Only human macrophages, monocytes and microglia – not rat or mouse cells – possess the proper CD4 molecule to bind gp120; however, lack of known receptors does not, of course, rule out alternative mechanisms of binding or toxicity. The macrophages might proteolyse gp120 and release a fragment of the molecule that is neurotoxic. Alternatively, the macrophages might release another toxic factor(s) that are damaging to neurons. One such macrophage factor might stimulate NMDA receptor-mediated neurotoxicity because NMDA antagonists block this form of neuronal damage[18]. However, activation of NMDA receptors by endogenous EAAs such as glutamate is necessary for the manifestation of gp120 neurotoxicity[29]. This finding raises the question of whether the macrophage toxic factor(s) might require a similar action of an endogenous glutamate-like agonist. If this were the case, then the macrophage toxic factor(s) itself would not necessarily have to interact directly with the NMDA receptor–channel complex. Obviously, further studies of the macrophage toxic factor(s) will be necessary to determine its site(s) of action. Recently, gp120 was reported to bind to galactosyl ceramide (GalC)[63,64], a molecule on the surface of oligodendrocytes (not illustrated). This finding raises the possibility that these glial cells may also be affected in the brains of patients with AIDS, perhaps disrupting myelin formation and indirectly injuring neurons.

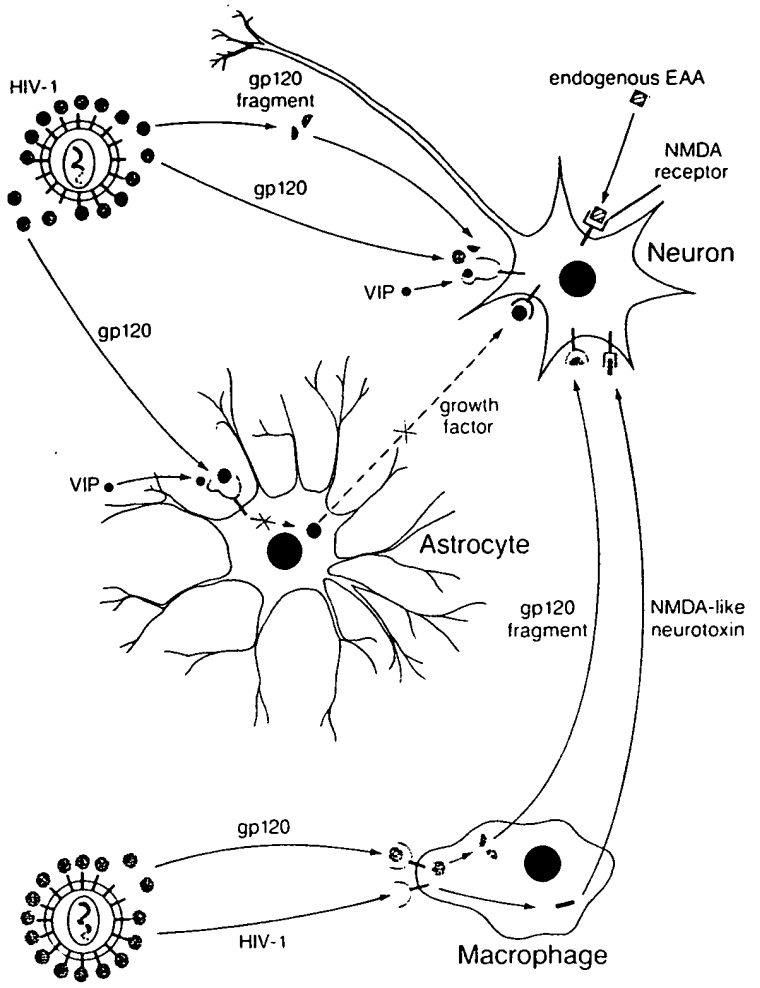

Fig. 1. *Schematic representation of hypotheses to explain HIV-related neurotoxicity. These theories are not all-inclusive but do serve to point out potential relationships among the findings of several laboratories as discussed further in the text. The virus and cell types are not drawn to scale.*

receptors. This hypothesis could probably account for many of the observed HIV-related neurotoxic phenomena, except for the fact that Giulian *et al.*[18] reported that quinolinate was not increased in the medium containing their macrophage toxic factor(s). Nevertheless, it will be important to determine if similar results are obtained from other culture systems; the quinolinate hypothesis is still worthy of further consideration.

It is also worth noting that NMDA receptor-mediated neurotoxicity appears to be involved in the action of other infectious agents. For example, MK-801 inhibits the neurotoxic effects of a hamster neurotropic strain of measles virus[51], as well as the destruction of nerve cells by tetanus toxin in rat hippocampus[52]. In addition, β-amyloid has been reported to enhance the neurotoxicity of L-glutamate *in vitro*[53]. Each of these observations is consistent with a possible final common pathway of neuronal injury involving EAA receptors in other infectious and neurodegenerative phenomena.

Although NMDA antagonists ameliorate gp120-induced neurotoxicity, inhibitors of the other types of EAA receptors (non-NMDA antagonists such as CNQX) are ineffective in affording protection from gp120 *in vitro*[29]. Similarly, NMDA, but not non-NMDA, antagonists block the lethal effects of the macrophage toxic factor(s) (see Table I)[18]. Preliminary experiments suggest that low (nanomolar) concentrations of L-type Ca^{2+} channel antagonists

TABLE I. Pharmacological profile for the prevention of HIV-related neurotoxicity by excitatory amino acid (EAA) antagonists

Insult	Attenuation of neurotoxicity by: NMDA antagonists	non-NMDA antagonists	Refs
The gp120 protein or a fragment of it	+	–	28, 29
Macrophage toxic factor(s)	+	–	18

such as nimodipine may not only prevent gp120-induced neurotoxicity[14], but also the deleterious consequences of factors secreted by HIV-infected macrophages [Pulliam, L., pers. commun., but see also Ref. 18, which reports adverse effects of higher (micromolar) concentrations of Ca^{2+} channel antagonists]. Overall, the similar profile of pharmacological protection from gp120 and from HIV-infected monocyte and macrophage products may possibly reflect the fact that at least one of the monocytoid toxic factors is somehow related to gp120.

Nevertheless, molecular-sieving and protease-digestion experiments suggest that the monocyte and macrophage toxic factor(s) does not appear to be intact gp120 (Ref. 18) – although a gp120 fragment remains a possibility, as does macrophage activation by gp120 to release the toxins. The toxic factor(s) may be as small as 300–400 Da (Giulian, D., pers. commun.). Alternatively, it is also plausible that macrophages secrete several neurotoxic factors unrelated to gp120, and that the similar pharmacologic profile of protection is a coincidence arising from the Ca^{2+}-dependent nature of a common final pathway to neuronal demise. Parsimony, however, favours a relationship between gp120 and the macrophage toxin(s).

Potential role of astrocytes

Astrocytes may also be important in mediating HIV-related neurotoxicity (Fig. 1). For example, in hippocampal cultures Brenneman *et al.*[13] have found that gp120-induced neurotoxicity can be prevented by the presence of vasoactive intestinal polypeptide (VIP) or by a five amino acid substance with sequence homology to VIP, peptide T. Brenneman and co-workers have also found that VIP acts on astrocytes to increase oscillations in intracellular Ca^{2+} and to release factors necessary for normal neuronal outgrowth and survival[54,55]. These results raise the possibility that gp120 may compete with endogenous VIP for a receptor, most likely on astrocytes, that is important for neuronal function. This effect of gp120 is hypothesized to prevent the release of astrocyte factors that are necessary to prevent neuronal injury, and suggests that one pathway for neuronal damage is an indirect one that is mediated through astrocytes.

Along these lines, Pulliam *et al.*[56] have recently reported that gp120 leads to decreased GFAP staining for astrocytes in human brain cell aggregates; ultrastructurally, cells resembling astrocytes lose their cytoplasmic fibrils. Thus, gp120 may cause alterations in astrocytes that could in turn conceivably lead to abnormal neuronal function. Finally, astrocytes have the capacity to produce quinolinate[57], but it is as yet unknown if gp120 might effect quinolinate release from astrocytes and produce yet another route for neuronal injury. In addition, following neuronal loss, astrocytes have been found to increase their synthesis of quinolinate, and therefore a feedforward pathway for neuronal injury could ensue from the resulting increase in quinolinate levels[58].

Concluding remarks

Toxic factors from HIV-infected human monocytoid cells may lead to neuronal damage *in vitro*. The HIV coat protein gp120 is also a neurotoxin *in vitro*, and preliminary data in rodents suggest that gp120 may be deleterious to neurons *in vivo* as well. It is as yet unknown if HIV-infected macrophage toxins include a gp120 fragment or, alternatively, if gp120 triggers the release of these neurotoxic factors. In the future, it will be important to determine which of these routes to neuronal damage *in vitro* actually contributes to the neurotoxicity observed *in vivo* in patients with HIV-1-associated cognitive/motor complex. At this juncture, it is tempting to speculate that gp120 or a fragment of this molecule may be responsible, at least in part, for some aspect of neuronal injury – but it would be premature to conclude this with any degree of certainty. This hypothesis has defined factors, can explain gp120 neurotoxicity by either direct or indirect routes, and requires the concurrent action of endogenous EAAs such as glutamate and quinolinate at NMDA receptors. Although this by no means would disprove other possible mechanisms for neuronal damage, it would represent at least one complete pathway whereby neurons might be injured in the brains of AIDS patients. Perhaps the most useful aspect of this hypothesis is that various treatment strategies may develop from this line of research, and they can be tested. Based upon studies *in vitro*, Ca^{2+} channel antagonists, NMDA antagonists, or VIP congeners represent promising forms of pharmacological intervention to protect neurons from HIV-related injury. In fact, safety testing and human trials are currently underway with some of these agents as basic research continues.

In summary, the results from several laboratories suggest that AIDS-related neuronal injury may be mediated by several pathways that most likely originate from toxins released by HIV-infected macrophages. There may be an intricate web of neurotoxic factors interacting with macrophages (or microglia), astrocytes and neurons. Nevertheless, this complex network may in the future be amenable to pharmacotherapy because of common final pathways of attack involving growth factors, NMDA receptors and deleteriously high levels of intracellular Ca^{2+}.

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Note added in proof: Recently, another HIV-1 protein, Nef, has been shown to affect neuronal cell function. Nef shares sequence and structural features with scorpion toxin peptides; both recombinant Nef protein and a synthetic portion of scorpion peptide increase total K^+ current in chick dorsal root ganglion cells [Werner, T. *et al.* (1991) *AIDS* 5, 1301–1308]. Another recent development concerns the characterization of at least some of the neurotoxic products of rodent brain macrophages. These cells have been shown to release glutamate, which results in NMDA-receptor-mediated neurotoxicity [Piani, D., Frei, K., Do, K. Q., Cuénod, M. and Fontana, A. (1991) *Neurosci. Lett.* 133, 159–162], and reactive oxygen intermediates, which damage neurons via H_2O_2 [Théry, C., Chamak, B. and Mallat, M. (1991) *Eur. J. Neurosci.* 3, 1155–1164].

Hans Kuypers Memorial Fund

Hans Kuypers was one of the pioneers of neuroscience. Ray Lund, Professor of Anatomy at the University of Cambridge has established a 'Hans Kuypers Memorial Fund' to commemorate the contribution of Hans Kuypers (1925–1989) to neuroscience. As a first step, the Fund will be used to support an Annual Lecture, plus honorarium and prize and, perhaps in the longer term, a Hans Kuypers research fellowship. In this way the name of Hans Kuypers will continue to be associated with new and exciting ventures in neuroscience.

All of those who were privileged to know him or work with him are fully aware of the really important contributions that he made during his lifetime. If you would like to support the Fund, please send cheques or money orders to: Prof. R. D. Lund, Dept of Anatomy, Cambridge University, Cambridge CB2 3DY, UK. Cheques etc. should be made payable to 'The University of Cambridge (Hans Kuypers Memorial Fund)'.

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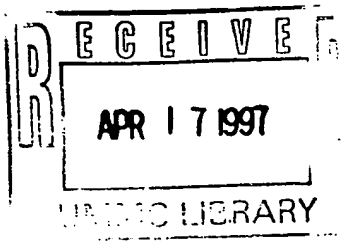

Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders?

F. Nicoletti, V. Bruno, A. Copani, G. Casabona and T. Knöpfel

Metabotropic glutamate (mGlu) receptors are a large, heterogeneous family of G-protein coupled receptors, which modulate excitatory synaptic transmission through various transduction pathways. Evidence is now accumulating that individual mGlu-receptor subtypes mediate distinct, facilitatory (group I subtypes) or inhibitory (group II and group III subtypes), actions on neurodegenerative processes. Drugs interacting with mGlu receptors are expected to influence both the induction and progression of neuronal degeneration without hampering the efficiency of fast excitatory synaptic transmission. For these reasons, mGlu receptors can be considered as promising drug targets in the experimental therapy of acute or chronic neurodegenerative diseases.

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GLUTAMATE, the principal excitatory transmitter in the mammalian brain, activates not only ionotropic glutamate (iGlu) receptors but also metabotropic glutamate (mGlu) receptors, which couple to G proteins and control the activity of membrane enzymes and ion channels[1,2].

An excessive activation of iGlu receptors induces large increases in the concentration of neuronal cytosolic free Ca^{2+}, due to Ca^{2+} influx through iGlu receptors that are Ca^{2+} permeable (in particular NMDA receptors) or secondary activation of voltage-operated Ca^{2+} channels (VOCC), or both. This will eventually result in neuronal death, via a pathophysiological process commonly designed as 'excitotoxicity'[3,4]. The prominent role of NMDA receptors in the pathophysiology of excitotoxic neurodegeneration, as occurs in stroke, hypoglycemic coma, cerebral trauma and subarachnoidal hemorrhage, has stimulated an intense search for antagonists of NMDA receptors (or other iGlu receptors) as neuroprotective agents. There are, however, some shortcomings associated with the therapeutic use of iGlu-receptor antagonists[5,6]. Inhibition of these receptors, for example, might compromise fast excitatory synaptic transmission and complicate chronic therapy in patients with a high risk for excitotoxic events; in addition, NMDA-receptor antagonists are particularly effective during the early induction phase of neuronal damage, whereas the therapy of acute brain ischemia requires drugs that retain their efficacy when administered several hours after the ischemic insult.

Metabotropic glutamate receptors are drug targets with the potential of overcoming these limitations, because they are expected to mediate excitatory synaptic transmission only under particular circumstances, for example during synaptic hyperactivity[7]. In addition, mGlu receptors modulate the activity of VOCC (see below), and affect some of the intracellular events that allow the progression of excitotoxic damage.

The mGlu-receptor family comprises eight subtypes, subdivided into three groups on the basis of sequence similarity and transduction pathways. Group I includes $mGlu_1$ (splice variants: a,b,c,d,e) and $mGlu_5$ (splice variants: a,b) receptors, which are coupled to polyphosphoinositide (PI) hydrolysis in heterologous expression systems[1,2,8]. A negative coupling of group I mGlu receptors to K^+ channels has been shown in several neuronal and non-neuronal preparations[9–12]. The $mGlu_{1a}$ receptor appears to be localized at the periphery of the postsynaptic membrane, as opposed to iGlu receptors, which occupy the 'core' of the synapse[13]. Thus, $mGlu_{1a}$ receptors are expected to be activated by excessive amounts of glutamate, which are released during synaptic hyperactivity. In addition, an mGlu receptor coupled to PI hydrolysis (the identity of which is unknown) is located at presynaptic level, and its activation enhances glutamate release in the presence of arachidonic acid[14].

Metabotropic glutamate receptors from group II ($mGlu_2$, $mGlu_3$) or group III ($mGlu_{4a}$, $mGlu_{4b}$, $mGlu_6$, $mGlu_{7a}$, $mGlu_{7b}$, $mGlu_8$) are negatively coupled to adenylate cyclase activity when heterologously expressed in non-neuronal cells (for reviews, see Refs 1,2). These receptors are also negatively coupled to VOCC (Refs 12,15,16). A presynaptic localization has been shown for some of the members of group II or group III mGlu receptors, including $mGlu_2$, $mGlu_4$ and $mGlu_7$ (Refs 2,17). Pharmacological activation of all these subtypes reduces glutamate release[18], whereas $mGlu_2$-receptor antagonists amplify the elicited release of glutamate[19]. Thus, $mGlu_2$ receptors at least (and perhaps other subtypes) might function as inhibitory autoreceptors[18]. Hence, it can generally be assumed that activation of group I mGlu receptors increases neuronal excitation and excitability, whereas activation of group II or group III mGlu receptors reduces synaptic excitation. However, there are exceptions. For example, in the CA1 region of the hippocampus agonists of

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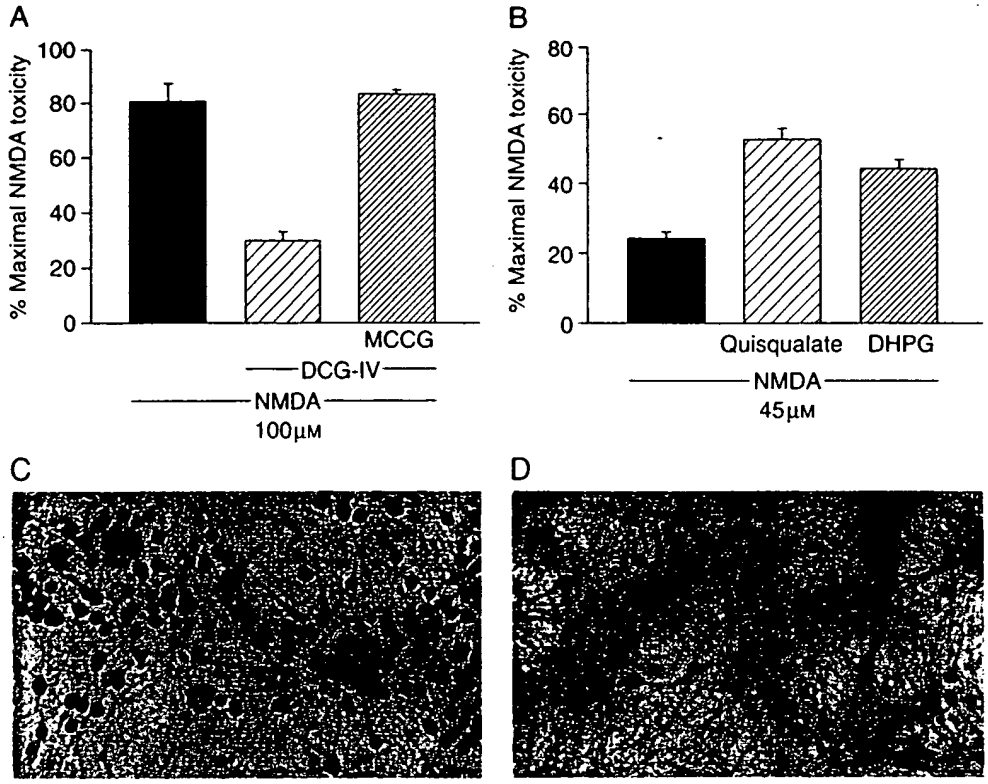

Fig. 1. *Opposite influence of group I or group II mGlu-receptor agonists on excitotoxic neuronal death. Mixed cultures of mouse cortical cells (prepared as described in Ref. 23) were exposed to a 10 min pulse with NMDA in the absence or presence of mGlu-receptor agonists. Neuronal degeneration was estimated by measuring the release of lactate dehydrogenase, and expressed as a percentage of the maximal NMDA toxicity (obtained by applying 300 μM NMDA). NMDA toxicity is prevented by the group II mGlu-receptor agonist (2s,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) (1 μM)* **(A)**, *or potentiated by the group I mGlu-receptor agonists, quisqualate (30 μM) or 3,5-dihydroxyphenylglycine (DHPG) (200 μM)* **(B)**. *The neuroprotective action of DCG-IV is antagonized by (2s,1's,2's)-2-methyl-2-(2'-carboxycyclopropyl)glycine (MCCG) (300 μM)* **(A)**. *In these cultures, neurons express both $mGlu_2$ and $mGlu_3$ receptors* **(C)** *and $mGlu_5$ receptors* **(D)**, *as indicated by positive immunostaining with specific antibodies[26]. The specificity of antibodies has been tested in HEK 293 cells transiently transfected with $mGlu_{5a}$- or $mGlu_2$-receptor cDNAs. Scale bars, 20 μm.*

group I mGlu receptors inhibit synaptic transmission through a presynaptic mechanism[20,21], whereas activation of group II mGlu receptors, located on GABA-containing nerve endings (inhibitory heteroreceptors), can eventually lead to neuronal disinhibition[18]. Apart from these particular cases, one expects that excitotoxicity is prevented by group I antagonists, while agonists of group II or group III are neuroprotective. The recent availability of group-specific agonists and antagonists of mGlu receptors allows investigation of these possibilities in more detail.

Activation of group II mGlu receptors is 'neuroprotective'

A series of cyclopropyl- and phenyl-glycine derivatives have recently been introduced as relatively selective agonists of group II mGlu receptors. Among these, (2S,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) and S-4-carboxy-3-hydroxyphenylglycine (4C3HPG) protect cultured neurons against neuronal degeneration induced by excitotoxic concentrations of NMDA (Refs 22–25; Fig. 1A,C) or kainate[22,23,27]. From a therapeutical standpoint, it is relevant that group II mGlu-receptor agonists attenuate neuronal degeneration induced by hypoxia combined with glucose deprivation[24], a condition that reproduces the hallmark features of ischemic neuronal damage. An important requirement for the therapy of stroke is that a drug retains a significant efficacy when administered several hours after the onset of the ischemic insult. Group II agonists might satisfy this requirement, because DCG-IV rescues cultured neurons even if applied after a toxic pulse with NMDA, when the NMDA channel blocker, MK801, loses its neuroprotective activity[23]. Unfortunately, DCG-IV is not appropriate for *in vivo* studies, because it behaves as an NMDA-receptor agonist at high concentrations[28]. A more promising compound is (2S,1'S,2'S,3'S)-2-(2-carboxy-3-methoxymethylcyclopropyl)-glycine (*trans*-MCG-I), which behaves as a potent and selective $mGlu_2$-receptor agonist, but is devoid of activity on NMDA receptors[29].

Inhibition of (1) cAMP formation[24,25], (2) VOCC activation or (3) glutamate release[23] might well account for the neuroprotective effect of group II mGlu-receptor agonists against excitotoxic degeneration. However, the ability of group II agonists to protect neurons against staurosporine-induced apoptotic death[30] cannot be simply related to any of these mechanisms, because staurosporine acts as a broad-spectrum inhibitor of cAMP-dependent protein kinase, Ca^{2+}–calmodulin kinase type II and protein kinase C (PKC). It is therefore possible that activation of group II mGlu receptors triggers a specific intracellular pathway that opposes the execution of programmed cell death. For example, the large amounts of βγ subunits released from G_i, the GTP-binding protein coupled to group II mGlu receptors, might activate the mitogen-activated protein (MAP) kinase pathway[31], thus inducing the expression of 'neuroprotective genes'. In astrocytes expressing $mGlu_3$, this process could eventually lead to the synthesis and secretion of neurotrophic factors, which support neuronal survival.

Recent evidence indicates that activation of group III mGlu receptors by the selective agonists L-2-amino-4-phosphonobutanoate (L-AP4) or L-serine-O-phosphate (L-SOP) also produces neuroprotective effects in cultured neurons or in brain slices[23,32]. These effects are prevented by (RS)-α-methyl-4-phosphonophenylglycine (MPPG) or (S)-α-methyl-3-carboxyphenylalanine (M3CPA)[33], which have recently been described as putative group III mGlu-receptor antagonists[34].

Inhibition of group I mGlu receptors has pros and cons

Stimulation of PI hydrolysis by excitatory amino acids is particularly elevated early after birth, and then declines progressively during postnatal development[35,36]. The receptor responsible for the early peak in PI hydrolysis has been now identified as the $mGlu_{5a}$

receptor, one of the two splice variants of the $mGlu_5$ receptor (Fig. 2A). The expression of $mGlu_{5a}$-receptor mRNA is higher in the early postnatal life than in the adult life when $mGlu_{5b}$-receptor mRNA becomes predominant instead[38]. In cultured cerebellar granule cells, apoptotic death induced by suboptimal growth conditions (that is, in medium containing low K^+) is observed after the developmental decline of $mGlu_5$-receptor activity (A. Copani, G. Casabona, V. Bruno, R. Kuhn, T. Knöpfel and F. Nicoletti, unpublished observations). In this system, apoptosis can be prevented by a continuous exposure to high concentrations of group I mGlu-receptor agonists (Fig. 2B), and is accelerated when an antagonist is applied during the early stages of granule-cell maturation[37]. Similar effects are shown in cultured cerebellar Purkinje cells, where a combined application of mGlu-receptor agonists and NGF promotes neuronal survival[39]. A role for group I mGlu receptors in neuronal survival has also been shown in studies *in vivo*. Inhibition of the mGlu receptors that are coupled to PI hydrolysis in infant rodents produces widespread degenerative effects in the retina and circumventricular organ zones[40]. The development of $mGlu_5$-receptor gene knockout mice will help clarify the role of this subtype in postnatal development.

Agonist-stimulated PI hydrolysis leads to mobilization of intracellular Ca^{2+} and activation of PKC (for review, see Ref. 41). While these intracellular events can provide a trophic support for developing neurons, they become potentially toxic if combined with additional mechanisms that lead to a sustained increase in intracellular Ca^{2+}. This point has been initially addressed in *in vivo* studies, in which mGlu receptors are shown to synergize with NMDA receptors in inducing neuronal damage[42,43]. In cultured neurons, drugs that preferentially activate group I mGlu receptors, such as quisqualate (in the presence of AMPA-receptor antagonists), s-3-hydroxyphenylglycine (3HPG) or 3,5-dihydroxyphenylglycine (DHPG) enhance NMDA toxicity[24,26] (Fig. 1B,D). 3HPG also aggravates neuronal damage induced by a mild hypoxia combined with glucose deprivation[24], suggesting that activation of group I mGlu receptors contributes to the pathophysiology of ischemic brain damage. Interestingly, the activity of group I mGlu receptors is upregulated in the hippocampus of rats that have been subjected to transient global ischemia[44,45]. There are some, as yet unsolved, issues concerning the excitotoxic effect of group I activation. For example, there are conditions in which activation of group I mGlu receptors protects against excitotoxic degeneration[46,47].

The ability of PKC to relieve the Mg^{2+} blockade of NMDA-gated ion channels[48] has been proposed as one of the fundamental processes by which activation of group I mGlu receptors amplifies NMDA toxicity[26]. This mechanism, which has been implicated in the facilitatory role of group I mGlu receptors on LTP (Refs 49,50), has been studied in detail in *Xenopus* oocytes co-transfected with the $mGlu_1$ receptor and individual NMDA-receptor subunits. Potentiation of NMDA currents by $mGlu_1$-receptor or PKC activation is greater in oocytes transfected with the NMDA-receptor subunits $N1_{100}$ and N2A than with those transfected with $N1_{100}$ and N2B, and is absent in oocytes transfected with $N1_{100}$ and N2C NMDA-receptor subunits[51,52]. Thus, the modulation of NMDA toxicity by mGlu receptors depends not only on the relative proportion of facilitatory and inhibitory mGlu-receptor subtypes, but also on the subunit composition of NMDA receptors.

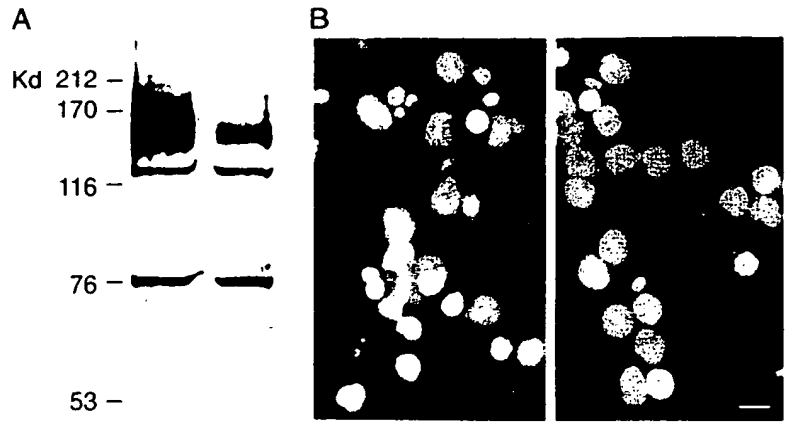

Fig. 2. *Group I mGlu receptors support neuronal survival during development.* **(A)** *Shows a western blot analysis performed in protein extracts from the cerebellum of rats at postnatal day 9 (left) or adult rats (right) using a polyclonal antibody common to $mGlu_{5a}$ and $mGlu_{5b}$ receptors[26].* **(B)** *Shows cerebellar granule cells cultured in low K^+-containing medium (10 mM instead of 25 mM K^+)[37] in the absence (left) or presence (right) of quisqualate [100 μM, applied only once at the second day* in vitro *in the presence of the AMPA-receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX)]. The typical chromatin abnormalities of apoptotic degeneration (fragmentation or condensation) are shown by fluorescent staining with the dye, Hoechst 33258 (Ref. 37). Scale bar, 6.5 μm.*

In summary, it appears that activation of group I mGlu receptors is required for normal development, but antagonism of these receptors might be neuroprotective in brain ischemia or in other forms of acute neuronal degeneration[53].

Chronic neurodegenerative diseases: a role for mGlu receptors?

Intrastriatal infusion of the endogenous NMDA-receptor agonist, quinolinic acid, induces a relative loss of medium-size spiny neurons with sparing of interneurons, similar to that observed in the early phase of Huntington's chorea[54]. Local injection of 4C3HPG (which behaves as an agonist of group II and an antagonist of group I mGlu receptors) protects striatal neurons against degeneration induced by quinolinic acid[55,56]. The vulnerable medium-size spiny neurons of the striatum express mainly $mGlu_5$ and $mGlu_3$ receptors[57], suggesting that the protective effect of 4C3HPG is mediated by these subtypes.

Recent evidence indicates that mGlu receptors are also potential drug targets for the therapy of Parkinson's disease. Overactive glutamate-containing afferents from the subthalamic nucleus (SThN) to the substantia nigra pars compacta (SNc) and the internal segment of the globus pallidus (GPi) could cause both a progressive excitotoxic loss of dopamine-containing neurons in the SNc (Ref. 58), and a hyperstimulation of GABA-containing neurons in the GPi, which leads to a reduction of motor activity (for review, see Ref. 59). A depression of glutamate-mediated synaptic activity has been obtained in the SNc by local application of the mGlu-receptor agonist, 1-aminocyclopentane-1s,3R-dicarboxylic acid (ACPD), suggesting the presence of presynaptic group II mGlu receptors, which reduce the release of glutamate from SThN efferents[60].

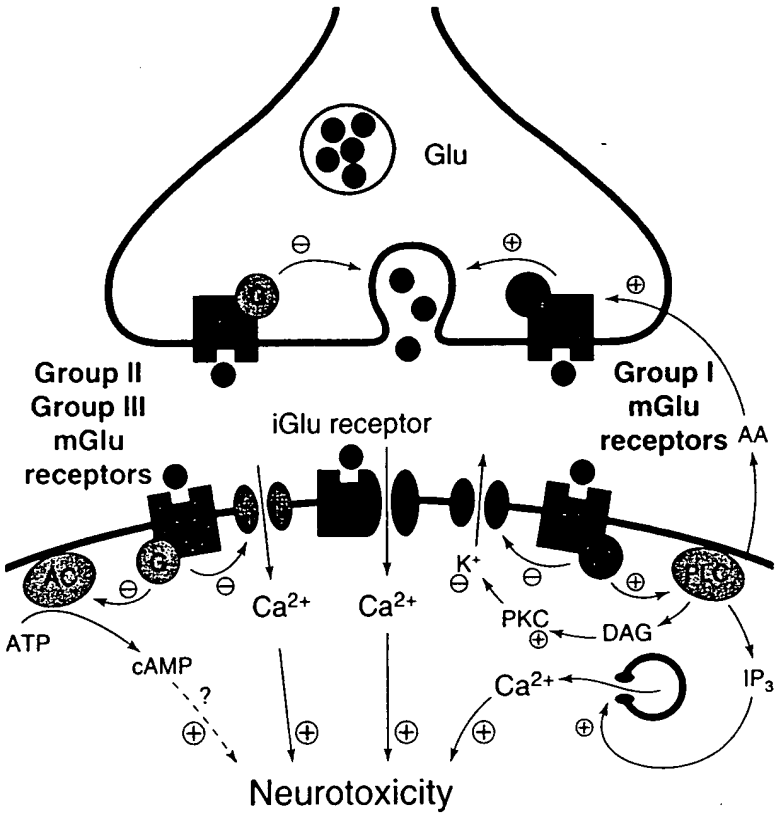

Fig. 3. *A scheme to illustrate the role of metabotropic glutamate (mGlu)-receptor subtypes on the induction and the progression of excitotoxic damage. At the presynaptic level, group II or group III mGlu receptors (green) inhibit glutamate (Glu) release, whereas putative group I mGlu receptors (red) enhance glutamate release. The latter effect requires the presence of arachidonic acid (AA), which acts as a retrograde messenger after being released from diacylglycerol (DAG) and membrane phospholipids. At the postsynaptic level, group II and group III mGlu receptors attenuate neuronal toxicity by reducing cAMP formation or the influx of extracellular Ca^{2+} through voltage-operated Ca^{2+} channels (other possible mechanisms are discussed in the text). Group I mGlu receptors, in contrast, amplify excitotoxic degeneration by either activating polyphosphoinositide hydrolysis or inhibiting different type of K^+ channels. Both activated protein kinase C and membrane depolarization are potentially able to enhance NMDA toxicity by removing the Mg^{2+} blockade of the NMDA-gated ion channel. Abbreviations: AC, adenylate cyclase; G, G protein; iGlu, ionotropic glutamate; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C.*

Activation of presynaptic mGlu receptors might at one time delay the degeneration of SNc neurons and improve motor activity.

Activation of mGlu receptors might also contribute to the pathophysiology of the slow neuronal degeneration observed in Macaques fed with β-M-methyl-N-amino-L-alanine (BMAA)[61], an excitotoxin present in the seed of the false sago palm *Cycas circinalis*, which behaves as a mixed agonist at iGlu and group I mGlu receptors[62–64]. Interestingly, BMAA has been proposed as one of the possible etiologic factors of a form of amyotrophic lateral sclerosis associated with parkinsonism and dementia, which was endemic in the Guam and Rota islands in the Western Pacific. Whether iGlu receptors and mGlu receptors synergize to cause neuronal damage in certain chronic degenerative disorders might be the subject of future investigation.

Activation of mGlu receptors might also interfere with the pathophysiological events underlying Alzheimer's disease. Activation of the $mGlu_{1a}$ receptor transfected into HEK 293 cells has been shown to increase the release of soluble forms of amyloid precursor protein (APPs)[65]. This effect, which might be accompanied by a reduction in β-amyloid production[66], is mimicked by the activation of other transmitter receptors coupled to PI hydrolysis and appears to be mediated by PKC. On the other hand, apoptosis induced by β-amyloid is substantially attenuated by agonists of group II and group III mGlu receptors[67], as well as by VOCC inhibitors[67,68]. The protective activity of mGlu-receptor agonists against apoptosis induced by β-amyloid might be related to their ability to reduce the influx of extracellular Ca^{2+} (see above), although whether or not β-amyloid peptide destabilizes the homeostasis of intracellular free Ca^{2+} is still a matter of controversy[69,70]. Therefore, activation of group I mGlu receptors might increase the production of APPs, thus reducing β-amyloid formation, whereas activation of group II mGlu receptors might protect neurons against the toxic effect of β-amyloid peptide.

TABLE I. Metabotropic glutamate (mGlu) receptors: pharmacology and putative role in neuronal toxicity

mGlu receptor subtypes	Transduction pathways	Agonists	Antagonists	Putative role in degeneration	Mechanisms
$mGlu_1$, $mGlu_5$ receptors (group I)	↑PI ↓I_K PLD?	3-HPG DHPG	UPF 531 (Ref. 73) 4-CPG CPCCOEt (Ref. 74)	Enhancement of neuronal toxicity	↑NMDA currents ↑Glu release Depolarization and VOCC opening
$mGlu_2$, $mGlu_3$ receptors (group II)	↓cAMP ↓I_{Ca} (L, N, P-VOCC) ↑I_K ↑cAMP ($Gi_{\beta\gamma}$?) (Ref. 71)	*trans*-MCG-I DCG-IV 4C3HPG	MCCG	Neuroprotection against excitotoxicity or apoptosis	↓cAMP ↓I_{Ca} ↓Glu release
$mGlu_4$, $mGlu_6$, $mGlu_7$, $mGlu_8$ receptors (group III)	↓cAMP ↓I_{Ca} (N, P-VOCC) (Refs 15,72)	L-AP4 L-SOP	MPPG	Neuroprotection?	↓Glu release

Abbreviations: L-AP4, L-2-amino-4-phosphonobutanoate; 4C3HPG, S-4-carboxy-3-hydroxyphenylglycine; CPCCOEt, cyclopropan[b]chromen-1a-carboxylic acid; 4-CPG, S-4-carboxyphenylglycine; DCG-IV, (2S,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, 3,5-dihydroxyphenylglycine; Glu, glutamate; 3-HPG, S-3-hydroxyphenylglycine; MCCG, (2S,1'S,2'S)-2-methyl-2-(2'-carboxycyclopropyl)glycine; *trans*-MCG-I, (2S,1'S,2'S,3'S)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine; MPPG, (RS)-α-methyl-4-phosphonophenylglycine; PI, polyphosphoinositide; PLD, phospholipase D; L-SOP, L-serine-O-phosphate; UPF 531, 1-aminoindan-1,5-dicarboxylic acid; VOCC, voltage-operated Ca^{2+} channel.

Concluding remarks

Several lines of evidence indicate that mGlu receptors influence the process of neuronal degeneration, and their activation might lead either to neurotoxicity or neuroprotection (see Fig. 3). Table 1 presents a 'state-of-the-art' view of the pharmacology of mGlu receptors and summarizes the role of individual mGlu-receptor subtypes in neurodegeneration, resulting mostly from cellular *in vitro* models. There is a clear indication for the potential use of selective group II mGlu-receptor agonists or group I mGlu-receptor antagonists as neuroprotective agents. These drugs could affect multiple steps in the chain of events that contribute not only to the induction, but also to the progression of neuronal damage. Based on the 'modulatory' function of mGlu receptors, we expect that group II mGlu-receptor agonists or group I mGlu-receptor antagonists have only a modest impact on fast excitatory synaptic transmission, which is undoubtedly a great advantage in chronic therapies. In addition, these drugs should be devoid of major peripheral side effects because, as opposed to other transmitter receptors that share the same transduction pathways (such as muscarinic, α_1 and α_2, 5-HT and neuropeptide receptors, etc.), mGlu receptors are not present in target organs of the autonomic nervous system. Thus, it appears that mGlu receptors satisfy some of the basic requirements to be considered as novel 'targets' in the experimental therapy of neurodegenerative disorders. Any further progress requires the availability of mGlu-receptor agonists or antagonists with greater potency, brain penetrance and subtype selectivity than those currently available.

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Erratum

In the article by R. Gerlai in the May issue of *TINS* (Vol. 19, pp. 177–180), Fig. 1 was incorrect. The F1 chromosomes of the wild-type offspring should contain one black chromosome and one grey chromosome, and the F2 chromosomes should have been labelled a–f from left to right. We apologize to the authors and the readers for this mistake.

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Function of metabotropic glutamate receptors in learning and memory

Gernot Riedel

Learning is the modification of behaviour by experience, and memory is the retention of such modifications. Since learning events might be of short duration, selective neuronal mechanisms must exist to translate transient activity into long-lasting memory. Because metabotropic glutamate (mGlu) receptors are coupled to various second messenger cascades they are ideal candidates for such translations. Their involvement in synaptic plasticity has been demonstrated recently, an important finding given that changes in synaptic efficacy are widely believed to be the physical substrate for information storage. Behavioural investigations using selective drugs have demonstrated that memory formation, especially of hippocampus-dependent tasks, is blocked by pre-training treatment with both mGlu-receptor agonists and antagonists. In contrast, agonists administered post-training might amplify memory formation. The hypothesis put forward here suggests that the primary function of mGlu receptors is to set the signal-to-noise ratio and thereby filter out unimportant or amplify important information.

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GLUTAMATE, the most abundant excitatory neurotransmitter in the CNS, activates a variety of receptors. Two principal types of glutamate receptors have been identified: (1) ligand-gated ion channels, including AMPA, kainate and NMDA receptors, which mediate fast excitatory transmission; and (2) G protein-coupled 'metabotropic' (mGlu) receptors, which indirectly regulate electric signalling and activate various second-messenger cascades. This latter property allows mGlu receptors to translate a short neuronal activation into long-lasting intracellular changes that are widely believed to underlie processes of learning and memory formation[1].

Distinct classes of mGlu receptors

Today, eight mGlu-receptor subtypes have been cloned (termed $mGlu_1$–$mGlu_8$; Ref. 2). Their deduced amino acid sequences indicate that mGlu receptors are much larger than, and do not show any homologies with, previously identified G protein-coupled receptors. Within the mGlu-receptor family, amino acid sequence identity varies and this has led to a classification into three classes[3]. The sequence identity within a class is about 70%, whereas the sequence identity between classes falls to about 40–45%. Class I comprises $mGlu_1$ and $mGlu_5$, class II comprises $mGlu_2$ and $mGlu_3$, and class III comprises $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ (Fig. 1). This classification is further supported by demonstration of different transduction mechanisms in the three classes. Expression of receptor subtypes in various cell lines has shown that class I receptors stimulate phospholipase C, as revealed by an increase in phosphoinositide turnover and Ca^{2+} release from internal stores[4,5]. In addition, activation of class I mGlu receptors leads to the formation of diacylglycerol (DAG) which then might activate protein kinase C. In contrast, class II and III mGlu receptors are negatively coupled to adenylate cyclase, thereby reducing the amount of cAMP (Refs 6,7). They differ, however, in their pharmacological profile against specific agonists.

Pharmacology of mGlu receptors

Several substances acting on mGlu receptors are available today[2], a small number of which have been investigated behaviourally. The most widely used agonist is 1-aminocyclopentane-1s,3R-dicarboxylic acid (ACPD), which is selective for mGlu receptors and devoid of any activity against ionotropic glutamate receptors. ACPD has been shown to act as an agonist on class I mGlu receptors in brain slices[8,9]. Moreover, ACPD reduces forskolin-stimulated cAMP formation[10,11] confirming its potency against class II and class III mGlu receptors. More selective agonists have been described recently. *Trans*-azetidine-2,4-dicarboxylic acid (tADA) has been identified as a novel rigid glutamate analogue[12], and it has been reported independently by two groups that tADA selectively activates class I mGlu receptors in hippocampal slices[13] and mixed cortical-cell cultures[14], having virtually no effect on forskolin-stimulated cAMP formation in either preparation. In Chinese hamster ovary cells expressing human $mGlu_2$, however, tADA also reduced forskolin-stimulated cAMP formation[15]. The most potent agonist of class II mGlu receptors is (2s,1′R,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) as revealed in brain slices, with very low activity on class I mGlu receptors. Unfortunately, DCG-IV also activates NMDA receptors[16]. Class III mGlu receptors are selectively activated by 2-amino-4-phosphonobutyrate (AP4) but AP4 is ineffective against class I and class II mGlu receptors[7,17]. Since AP4 depresses glutamate release from perforant-path synapses[18], class III mGlu receptors are widely believed to be located presynaptically, thereby acting like autoreceptors.

The most widely used mGlu-receptor antagonist known today is (R,S)-α-methyl-4-carboxyphenylglycine

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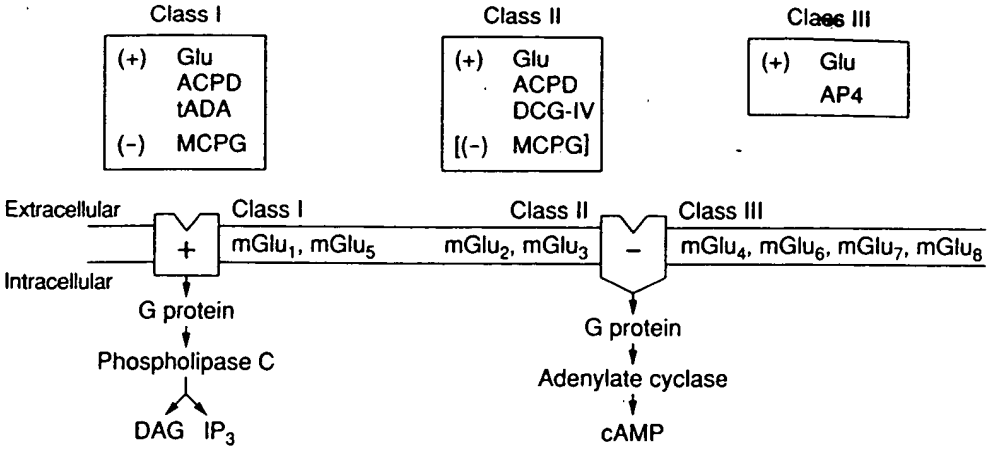

Fig. 1. *Classification of metabotropic glutamate (mGlu) receptors and their second-messenger cascades. Class I mGlu subtypes ($mGlu_1$ and $mGlu_5$) are positively (+) linked via G proteins to phospholipase C. Stimulation of this enzyme results in breakdown of membrane phospholipids into the chemical messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from internal stores. Various agonists and antagonists have been identified for class I receptors, of which ACPD and tADA as well as MCPG have been investigated behaviourally. Class II and class III mGlu receptors are negatively (–) coupled to G protein-mediated activation of adenylate cyclase. This action causes depression of the chemical messenger cAMP. In learning experiments, ACPD and DCG-IV have been investigated as class II agonists. Selective antagonists for these classes have not yet been examined. Abbreviations: ACPD, 1-aminocyclopentane-1s,3R-dicarboxylic acid; AP4, 2-amino-4-phosphonobutyrate; DCG-IV, (2s,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; Glu, glutamate; MCPG, (R,S)-α-methyl-4-carboxyphenylglycine; tADA, trans-azetidine-2,4-dicarboxylic acid.*

(MCPG; Ref. 19) with the (+)-isomer being the active form[20] (for contrary results, see Ref. 21). Although it has been reported as a competitive antagonist against $mGlu_1$ and $mGlu_2$ expressed in cell lines[22], we have found that MCPG inhibits agonist-stimulated phosphoinositide hydrolysis in a dose-dependent manner in hippocampal slices, but does not block the ACPD-induced depression of forskolin-stimulated cAMP formation[11] indicating that, at least in the hippocampus, MCPG might be selective for class I mGlu receptors.

mGlu receptors in learning and memory

The importance of glutamate receptors in learning and memory in rats has been convincingly demonstrated by Morris and colleagues in the water maze[23,24]. They found that blockade of the NMDA receptor by means of intracerebroventricular (i.c.v.) minipump injection of 2-amino-5-phosphonopentanoate (AP5) impaired the formation of spatial memory needed to find a hidden platform in the pool, but had no effect on visual discrimination in the same environment. Moreover, hippocampal long-term potentiation (LTP), a phenomenon widely used as a model to investigate the cellular processes involved in learning and memory, was also abolished in the presence of AP5 (Refs 23,24). Early research on mGlu receptors has focused on LTP and repeatedly demonstrated that mGlu receptors play an important part in hippocampal LTP, both *in vitro*[25–27] and in freely moving animals[11,28,29]. However, some experiments have failed to block LTP in the presence of MCPG (Refs 30–32), which might be due to different experimental conditions.

The involvement of mGlu receptors in LTP has encouraged the investigation of their role in a variety of behavioural paradigms using various injection protocols.

Pre-training injection

In most behavioural studies on mGlu receptors, agonists or antagonists are injected prior to training. The first of these reports found that i.c.v. infusion of the antagonist MCPG disrupted memory formation in a spatial-alternation task[33] performed in a newly developed shock-reinforced Y-maze[34]. Rats were trained 30 min after treatment to escape foot shock in the start box by running into the goal box in a right–left–right–left sequence. Rats exposed to MCPG showed no deficits during acquisition training, but were amnesic when tested for retention 24 h later (Fig. 2A; Ref. 33). Similar retention deficits have subsequently been reported in water-maze learning in rats[35] and in passive-avoidance learning in young chicks presented with a bead dipped in methylanthranylate causing a clear disgust response[36,37]. The memory block is dose dependent, and a brain concentration of 500 μM of (+/–)-MCPG is required for a maximal block of memory formation in chicks[37] and rats[38]. Moreover, injections of MCPG immediately prior to retention testing in the Y-maze task did not affect recall[33,38] suggesting that activation of mGlu receptors might be required during and shortly after learning, but not during retention. MCPG treatment prior to acquisition training did not affect the formation of memory in rats trained either in the open field[39] or in a shock-reinforced brightness-discrimination paradigm (Fig. 2C; Ref. 38). This pattern of results suggests that spatial-learning tasks are peculiarly sensitive to mGlu receptors.

Application of mGlu-receptor agonists prior to tetanic stimulation has been shown to facilitate and prolong LTP, both in hippocampal slices and *in vivo*[27,29]. Thus, one would expect similar actions on memory formation, but learning experiments provide contrary results. In the water maze, ACPD caused an impairment of acquisition[40] suggesting that pre-training activation of mGlu receptors might interfere with the process of learning. However, no test for memory was conducted under drug-free conditions, and it remains unclear whether ACPD treatment might also cause a memory deficit. This is likely, because i.c.v. injections of the agonist tADA, which caused no performance deficit during acquisition, produced amnesia in spatial-alternation (Fig. 2B), but not in brightness-discrimination learning (Fig. 2D; Refs 38,41). Given that tADA preferentially activates class I mGlu receptors[13,14], it remains to be determined whether the learning impairment under ACPD was due to the additional activation of other mGlu-receptor subtypes.

In line with this hypothesis, recent electrophysiological data indicate that stimulation of class II and class III mGlu receptors leads to a depression of neuronal excitation[42,43]. Such depressions might cause unspecific behavioural effects. In fact, i.c.v. injection of ACPD in rats has been reported to increase the level of activity during performance in the water-maze task[40]. Furthermore, in an elegant study Nakanishi and co-workers have provided compelling evidence for the behavioural relevance of the depression of neuronal excitation via class II mGlu receptors[44]. While mating, pheromones of the male are important in order to form an olfactory memory in female mice. Subsequent

exposure to the pheromone of a strange male then causes pregnancy block, whereas exposure to the same pheromone does not. This memory appears to depend on synaptic changes in the olfactory bulb, in which $mGlu_2$ is expressed predominantly at granule-cell dendrites. Selective activation of $mGlu_2$ by means of DCG-IV or ACPD injections in the presence of male pheromones has been shown to induce olfactory memory without the occurrence of mating[44]. Memory formation depends on prolonged $mGlu_2$ activation, thereby depressing the GABA-mediated feedback inhibition from granule cells to mitral cells[44]. Possibly because of its weak potency against $mGlu_2$ (Refs 11,21) MCPG failed to inhibit memory formation in female mice[44].

Post-training injections

In young chicks, passive-avoidance learning is disrupted by intracerebral injection of the antagonist MCPG at 5 min, but not at 15 min, postacquisition[37]. Likewise, intrahippocampal administration of MCPG in rats immediately after training in a one-trial step-down avoidance procedure caused amnesia (Fig. 3A), but had no effect if administered 180 min after training[45]. These results indicate that early phases of memory formation are MCPG-sensitive, and might thus depend on mGlu-receptor modulation. However, rats injected with MCPG immediately post-training did not perform any differently to the vehicle-treated controls when trained in a Y-maze for spatial alternation or brightness discrimination (Fig. 3B and C; Ref. 38). Several plausible explanations could account for this discrepancy. First, i.c.v. injections require longer times for the drug to diffuse to the target site, whereas intracerebral applications allow the compound to act instantaneously. Second, the training procedure in the Y-maze, which takes 30–40 min, might be too long, so that early MCPG-sensitive processes of memory formation are already completed. Finally, since controls, which have been trained on spatial alternation, and which received injections of saline immediately post-training, were amnesic, the effect of MCPG could not be assessed properly under these conditions.

In rats, post-training application of mGlu-receptor agonists facilitate retention compared with saline-injected controls[38,45]. In the passive-avoidance task, intrahippocampal administration of ACPD potentiated retention in a dose-dependent fashion (Fig. 3A; Ref. 45) suggesting that a brief activation of the mGlu receptor after a short learning event facilitates retention. Similarly, i.c.v. injections of tADA immediately post-training enhanced retention of a spatial-alternation, but not a brightness-discrimination task (Fig. 3B and C). However, post-training i.c.v. injection of saline attenuated memory formation, and thus the enhancement obtained with tADA is a facilitation only with respect to amnesic controls[38]. Given the specificity of tADA, these data support the idea that it might be the post-training activation of the mGlu receptors coupled to phospholipase C ($mGlu_1$ and $mGlu_5$) that results in memory enhancement.

$mGlu_1$ mutant mice

Recent advances in molecular biology techniques have led to creation of mutant mice lacking functional $mGlu_1$ (Refs 46–48). Learning impairments have been reported during acquisition in the water maze[46] and eyeblink conditioning[48], but not during contextual fear conditioning[47]. Retention of the fear-conditioned

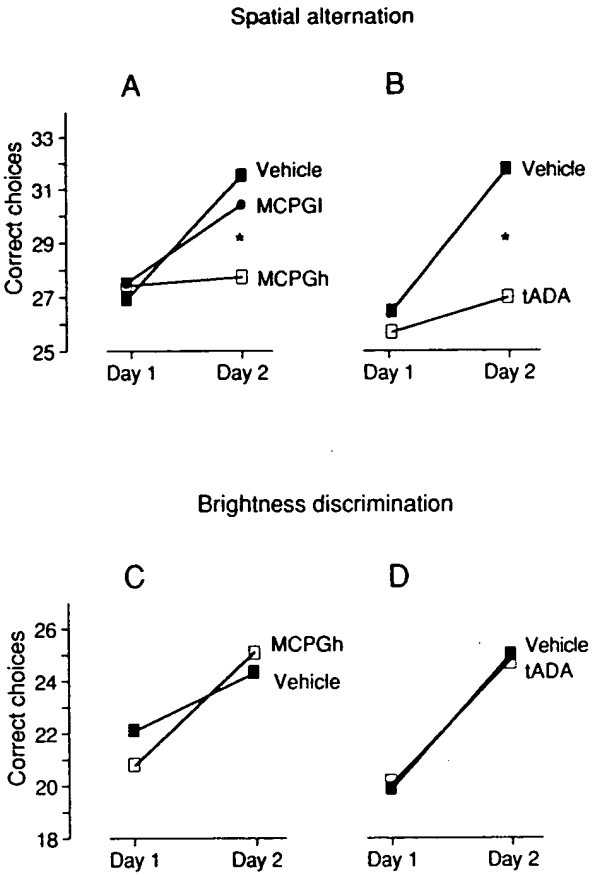

Fig. 2. ***Pre-training i.c.v. injections of the mGlu-receptor antagonist MCPG or agonist tADA causes amnesia in spatial-alternation but not brightness-discrimination learning in rats.*** *The graphs show mean data, expressed as the number of correct choices per session. Drugs (vehicle = saline 0.9%, MCPGl = 20 mM, MCPGh = 200 mM and tADA = 20 mM in 5 μl each) were applied i.c.v. 30 min prior to training (day 1) and animals were tested for retention 24 h later (day 2).* **(A and B)** *show performance in spatial-alternation training (40 trials per session). Only MCPGh (calculated concentration, 500 μM, assuming a uniform distribution and a brain volume of 2 ml) and tADA (calculated brain concentration 50 μM) prevent memory formation, whereas animals treated with MCPGl (calculated brain concentration, 50 mM) behave like vehicle-injected controls.* **(C and D)** *demonstrate that brightness-discrimination learning (30 trials per session) was not altered by pre-training i.c.v. infusion of MCPGh or tADA. Asterisks indicate significant group differences compared with vehicle-treated controls as revealed by ANOVA ($P<0.05$). Abbreviations: i.c.v., intracerebroventricular; MCPG, (R,S)-α-methyl-4-carboxyphenylglycine; MCPGh, high concentration of MCPG; MCPGl, low concentration of MCPG; tADA,* trans*-azetidine-2,4-dicarboxylic acid; mGlu, metabotropic glutamate.*

response, however, was significantly reduced in $mGlu_1$ mutant mice[47]. Since these animals showed severe deficits in motor co-ordination as revealed by various versions of rotorod tests[48], behavioural data have to be interpreted with some caution and further work should determine whether deficits in mutant animals are due to genuine learning impairment or some other developmental alteration.

Electrophysiological data on $mGlu_1$ mutant mice have demonstrated attenuation or complete block of cerebellar long-term depression[46,48], another form of long-lasting synaptic plasticity. A reduction of LTP has been reported in the hippocampal CA1 area in $mGlu_1$ mutant mice by Aiba and colleagues[47], but normal LTP in CA1 and dentate gyrus has been observed by Conquet and co-workers[46].

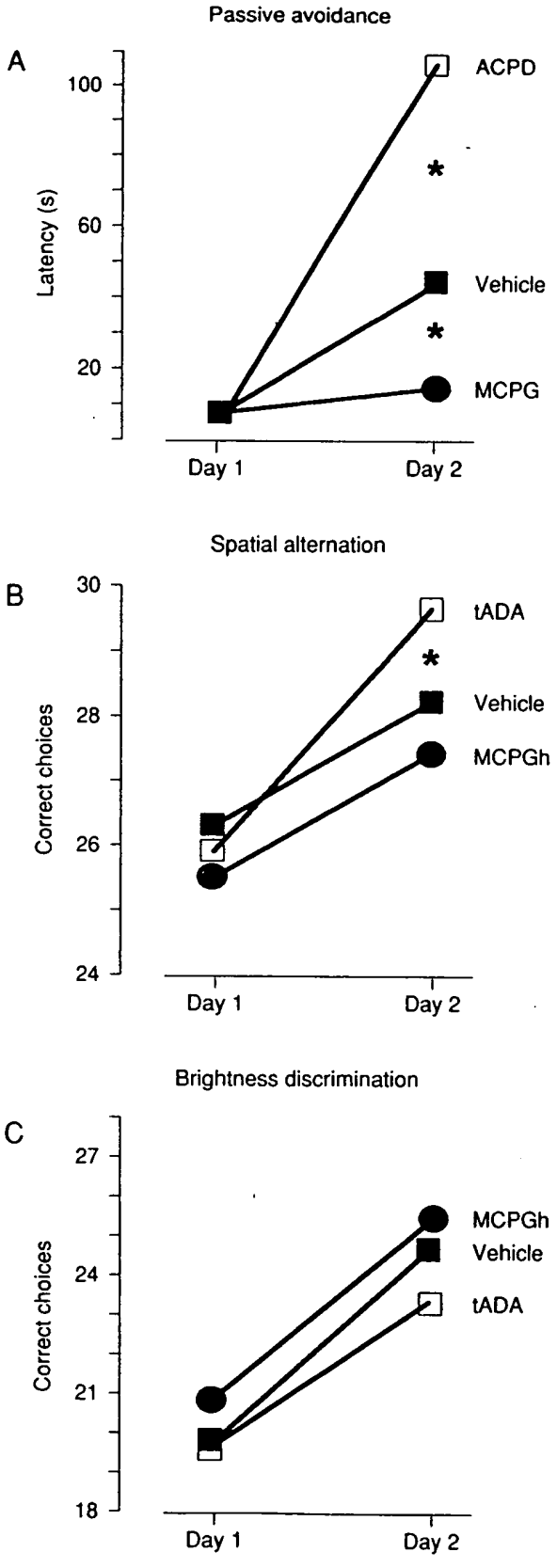

Fig. 3. *Post-training injection of mGlu-receptor agonists enhance retention of hippocampus-dependent learning tasks. Retention of a passive-avoidance paradigm* **(A)** *is amplified by post-training (day 1) intrahippocampal injection of ACPD (2.5 mg in 1 μl) compared with vehicle-injected controls, measured as increased latency to step down from a platform onto a shock-equipped grid floor (data from Ref. 45). Administration of MCPG (2.5 mg in 1 μl) causes amnesia. Similarly, tADA (assumed brain concentration, 50 μM) injected i.c.v. immediately post-training enhanced retention (day 2) of spatial-alternation* **(B)** *but not brightness-discrimination learning* **(C)**. *MCPGh (assumed brain concentration, 500 μM) had no influence on either task compared with vehicle-injected controls. The graphs show mean data, expressed as the number of correct choices per session. Asterisks indicate significant group differences compared with vehicle-treated controls as revealed by ANOVA ($P<0.05$). Abbreviations: ACPD, 1-aminocyclopentane-1s,3R-dicarboxylic acid; i.c.v., intracerebroventricular; MCPG, (R,S)-α-methyl-4-carboxyphenylglycine; MCPGh, high concentration of MCPG; mGlu, metabotropic glutamate; tADA,* trans-*azetidine-2,4-dicarboxylic acid.*

Towards a role of mGlu receptors in learning and memory

Our current understanding of the function of the mGlu receptor in cellular mechanisms of learning and memory is fragmentary. The observation that a whole family of these receptors with different pharmacological profiles exists in the CNS suggests distinct roles for certain mGlu-receptor subtypes. Their heterogeneous distribution in the brain supports this view. However, three tentative conclusions can be drawn from the reports available to date of the role of mGlu receptors in behaviour: (1) it appears that not all forms of learning involve mGlu-receptor activation, for example, brightness-discrimination learning in rats[38]; (2) in other learning paradigms, namely passive avoidance, spatial water-maze acquisition or spatial alternation in the Y-maze, which are believed to be hippocampus-dependent, mGlu-receptor modulation seems to be a prerequisite for memory formation. Evidence came from the observation that blockade or activation of mGlu receptors during learning causes amnesia[33,35,38], but that learning within a session is not affected (for contrary results see Ref. 40); and (3) in such learning situations the activation of mGlu receptors is limited to a time window during and shortly after acquisition learning. Blocking mGlu receptors hours after the learning event does not interfere with memory formation[45], nor are mGlu receptors involved in memory recall[33,35,38].

How could mGlu receptors participate in memory formation? The hypothesis put forward here is that the principal role of mGlu receptors during behaviour is to modulate the signal-to-noise ratio of the CNS. Under control conditions spontaneous neuronal activity is supposed to constitute a 'noise'. During a particular learning event, activation of mGlu receptors might produce a signal, and this increase in the signal-to-noise ratio is transformed into memory (Fig. 4A). Blockade of mGlu receptors, for example by MCPG, prevents changes in the signal-to-noise ratio and consequently leads to amnesia (Fig. 4B; Refs 33,35). Agonists, on the other hand, appear to have multiple effects on memory formation depending on the time of application. Pre-training injection might activate mGlu receptors nonspecifically, and result, as indicated for ACPD and tADA, in an increase in noise (Fig. 4C). Whether such activation is maximal apparently depends on the concentration of the drug. If the learning event occurs on the background of high noise levels, further stimulation of mGlu receptors is prevented, as is the formation of memory (Fig. 4C; Refs 38,40,41). It has been demonstrated electrophysiologically that the non-specific activation of mGlu receptors in hippocampal[49] as well as piriform cortical neurones (M. Fejtl *et al.*, unpublished observation) might be excitatory. Activation of class I mGlu receptors appears to depolarize

CA1 neurones, probably mediated by increases in phosphoinositide hydrolysis[50] or direct inhibition of various K^+ channels[51]. Other mGlu receptors might reduce the noise and thereby generate signals[44]. Stimulation of class II and class III mGlu receptors preferentially depresses excitation due to the inhibition of voltage-activated Ca^{2+} channels[51] or presynaptic reduction of transmitter release[52]. In contrast, a training-induced signal would be enhanced by post-training application of mGlu-receptor agonists, thereby amplifying memory formation (Fig. 4D; Refs 38,45). Training might induce a signal that is similar to the one found in controls (see Fig. 4A). Subsequent pharmacological activation of mGlu receptors using specific agonists is supposed to further increase this signal by specifically stimulating those receptors that have been activated in a use-dependent manner during training. Such use-dependent mGlu-receptor stimulations might cause an increase in receptor sensitivity against agonists, which prevents a simultaneous increase in noise. In fact, spatial learning in the eight-arm radial maze and classical conditioning has been shown to potentiate the amount of agonist-induced phosphoinositol hydrolysis in rat hippocampal slices and synaptosomes, respectively[53,54]. These data indicate that mGlu receptors could switch their sensitivity in a use-dependent manner, and indeed, such changes can have physiological consequences, as has been recently reported for LTP (Ref. 55).

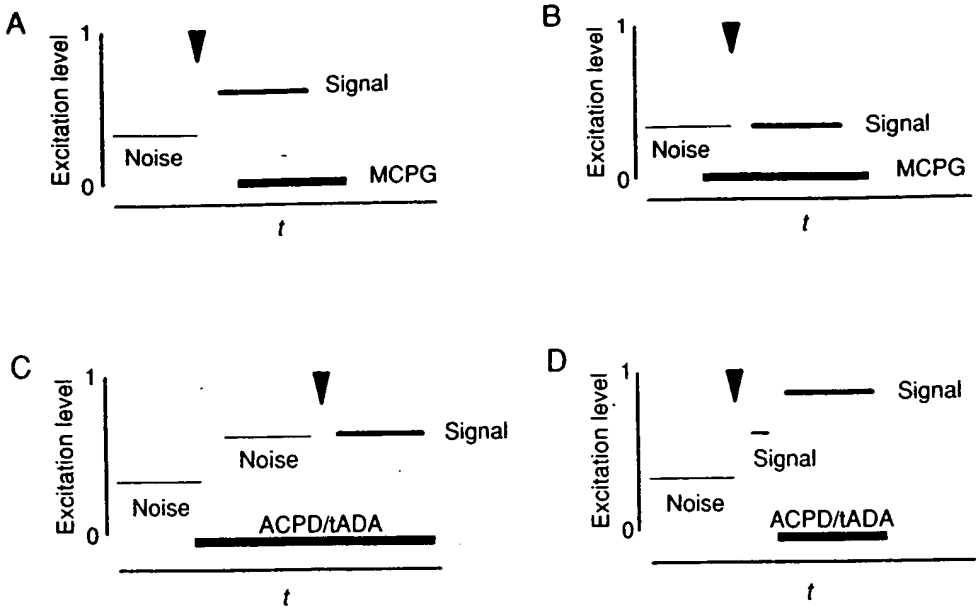

Fig. 4. ***Model for the role of mGlu receptors in behaviour: modulation of the signal-to-noise ratio.*** *The excitation level is hypothetically defined to be between 0 and 1. Arrowhead indicates the learning event (training). Spontaneous activity of neurones is assumed to constitute a 'noise', and stimuli too weak to generate a signal are filtered out.* **(A)** *During training the signal-to-noise ratio is shifted towards an increase in the signal, which then results in memory formation. Late block of mGlu receptors by MCPG does not affect retention.* **(B)** *Pre-training application of MCPG blocks the learning-induced shift of the signal-to-noise ratio and thereby prevents memory formation.* **(C)** *Agonists of class I mGlu receptors (ACPD or tADA) injected prior to training are assumed to increase noise nonspecifically. On the background of this high noise level, training-induced signals might not cause further enhancement of the excitation level. All that remains is a high noise level, which consequently leads to amnesia. Class II agonists (DCG-IV), on the other hand, are supposed to be capable of reducing the noise and thereby facilitate signals (not shown, see Ref. 44).* **(D)** *By way of activating mGlu receptors, acquisition training is supposed to constitute a signal that is similar to the one in controls (see Fig. 4A). Post-training administration of class I agonists (ACPD or tADA) might be capable of further enhancing the training-induced signal and thus lead to an increase in memory formation. The simultaneous nonspecific activation of mGlu receptors is prevented (for details, see text). Abbreviations: ACPD, 1-aminocyclopentane-1s,3R-dicarboxylic acid; DCG-IV, (2s,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; MCPG, (R,S)-α-methyl-4-carboxyphenylglycine; mGlu, metabotrophic glutamate; tADA,* trans*-azetidine-2,4-dicarboxylic acid.*

The model outlined here is in agreement with current knowledge and allows a variety of predictions which could be tested in the following way: (1) more selective pharmacological tools: a prerequisite for a better understanding of the function of particular mGlu-receptor subtypes is the availability of subtype-specific drugs. Such compounds are beginning to emerge and further work will benefit from their use. Investigations are expected to gain insight into the differential roles of mGlu-receptor members; (2) behavioural investigations – constant stimulus strength, various drug concentrations: the model predicts that certain concentrations of drugs are required in order to fully block or stimulate mGlu receptors. Preliminary evidence has been provided by experiments showing that the application of low concentrations of MCPG to rats has little effect on their memory (Fig. 3A), whereas rats injected with a tenfold higher concentration of MCPG show amnesia. According to the model, animals treated with intermediate drug concentrations, which have not been tested to date, would be expected to exert retention deficits that directly correspond to the amount of blockade or stimulation of mGlu receptors, but should not be amnesic; and (3) behavioural investigations – various stimulus strengths, constant drug concentration: if the major role of mGlu receptors is to modulate the signal-to-noise ratio, behavioural experiments could be designed in which a weak concentration of drugs might block memory formation. This block is achieved only if the strength of the stimulus occurring during acquisition training is weak, 0.3 mV shock intensity, for example. If the stimulus, however, increased, for example by the application of shock intensities of 1 mV, the hypothesis would predict memory formation to take place.

The model developed here is based on a limited set of reports. It is put forward in the hope that it will encourage further work on the function of mGlu receptors in learning and memory.

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PERSPECTIVES ON DISEASE

Apolipoprotein E, memory and Alzheimer's disease

Hilkka S. Soininen and Paavo J. Riekkinen Sr

Apolipoprotein E (ApoE) ε4 is a well-documented risk factor for Alzheimer's disease (AD). Patients with AD show neuronal damage, particularly in the medial temporal lobe structures involved in memory processing. ApoE has been implicated in nerve regeneration following injury, and synaptogenesis in the hippocampus of experimental animals. Recent studies have shown an increased accumulation of βA4 amyloid and an increased deficit in ACh-containing neurons in the brains of AD patients that are homozygous for ApoE ε4 compared with those lacking ε4. Furthermore, AD patients with two ApoE ε4 alleles have more-severe loss in hippocampal volume in magnetic resonance imaging (MRI) scans, and more impairment in tests assessing delayed memory, than AD patients without the ε4 allele, in spite of similar global severity of dementia. Minor changes in hippocampal MRI volumetry can also be detected in nondemented elderly, particularly in those with an ε4/4 genotype. Data from a population-based study revealed that elderly subjects carrying the ε4 allele had worse learning ability than those with the ε2/2 or ε2/3 phenotypes, whereas these groups did not differ in other cognitive domains. These data suggest that ApoE ε4 might influence the magnitude of medial temporal lobe atrophy and memory impairment in AD and also in nondemented elderly.

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ALZHEIMER'S DISEASE (AD) is a heterogeneous entity. This might entail differences in patient response to various drug treatments. One factor that might contribute to the heterogeneity of AD is the apolipoprotein E (ApoE) genotype. The ApoE ε4 allele is a well-established risk factor for AD (Refs 1–2). ApoE is a plasma protein that is involved in the transport of cholesterol and other lipids. It is implicated in the growth and regeneration of nerves during development or following injury[3]. ApoE is present in senile plaques, neurofibrillary tangles and cerebrovascular amyloid, the major neuropathological changes in AD

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Exhibit Q (10/644,645)

Activation of the metabotropic glutamate receptor attenuates *N*-methyl-D-aspartate neurotoxicity in cortical cultures

(*trans*-1-aminocyclopentyl-1,3-dicarboxylic acid/carbachol/Alzheimer disease/development/neurodegeneration)

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ABSTRACT **Excitatory amino acid receptor-mediated neurotoxicity (excitotoxicity) has been proposed to contribute to neuronal loss in a wide variety of neurodegenerative conditions. Although considerable evidence has accumulated implicating *N*-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in the processes of excitotoxicity, relatively little research has focused on the ability of other neurotransmitter systems to influence excitotoxic neuronal injury. In the present study, we examined the effects of *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD), a selective agonist for the metabotropic glutamate, or ACPD, receptor, and carbachol, an agonist at the acetylcholine receptor, on neuronal degeneration produced by brief exposure to NMDA in murine cortical cultures. Since excitotoxic neuronal injury is probably caused by increases in intracellular Ca^{2+} concentrations, the two transmitter agonists were of particular interest as both have been shown to mobilize intracellular calcium stores. Contrary to what might be expected, ACPD and, to a lesser degree, carbachol attenuated NMDA neurotoxicity. The neuroprotective effect of ACPD, but not of carbachol, was dependent upon the developmental state of cultures; in older cultures (≥18 days *in vitro*), the protective effect decreased. The neuroprotection by ACPD may be, in part, mediated by protein kinases, since protection is partially reversed by the protein kinase antagonists H-7 and HA-1004. These data suggest that concomitant activation of the ACPD receptor may serve as a protective mechanism against neurotoxicity that could be produced by brief intense NMDA receptor activation during normal or abnormal brain function.**

Excitatory amino acid (EAA) neurotoxicity (excitotoxicity) has been proposed to contribute to neuronal loss in a broad spectrum of neurodegenerative conditions including ischemia, Huntington disease, and Alzheimer disease (AD) (1, 2). As neurodegenerative diseases are known to be associated with a variety of specific pathologies, understanding the factors that can alter EAA neurotoxicity may prove clinically important. For example, β/A4 protein that accumulates in amyloid plaques of AD patients increases the vulnerability of cortical neurons to EAA-mediated neuronal injury (3). Similarly, other neurotransmitter systems, which are often altered anatomically or functionally in various disease states, could affect the susceptibility of neurons to excitotoxic damage. For instance, in AD, there is widespread reduction in cholinergic (4) and somatostatinergic markers (5, 6). The ability of the non-EAA system to affect EAA-mediated injury may reflect transmitter interactions in normal brain, as serotonin has been reported to change electrophysiological responses of neurons to *N*-methyl-D-aspartate (NMDA) (7, 8).

It is now well established that there are at least three general classes of glutamate receptors (9). The NMDA and the non-NMDA receptors (i.e., α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and kainate receptors) are directly associated with the specific ligand-gated ionophores that permit influx of cations upon activation (ionotropic receptor). In contrast, the recently discovered metabotropic glutamate, or *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD), receptor (10–12) activates a phospholipase, via a guanine nucleotide-binding regulatory protein, that hydrolyzes membrane phospholipid to generate the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG, in combination with Ca^{2+}, activates protein kinase C (PKC) (13), whereas IP_3 mobilizes Ca^{2+} from intracellular stores in various cell types (14).

Considering the evidence that calcium ions may play a critical role in producing excitotoxic neuronal damage (15, 16), each of the three receptors appears capable of contributing to neuronal damage by increasing intraneuronal free calcium concentrations. While activation of the ionotropic receptors is sufficient to produce most of the EAA neurotoxicity (1, 17, 18), probably through a large influx of calcium (15, 16, 19), evidence for involvement of the metabotropic receptor in excitotoxic neuronal damage was not available until recently, largely due to the lack of selective agonists or antagonists. In this regard, certain unusual pharmacological effects of a nonselective agonist such as quisqualate have often been difficult to interpret. For example, Garthwaite and Garthwaite (20) proposed that the 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX)-insensitive induction of CNQX-sensitive neurotoxicity by a pulse exposure to quisqualate may be mediated by the metabotropic receptor. However, a similar quisqualate neurotoxicity in cortical cultures has been shown to be largely attributable to the cellular uptake and subsequent release of quisqualate (21). The recent discovery of ACPD, a selective agonist for the metabotropic receptor (12), now makes it possible to directly examine the role of the ACPD receptor in excitotoxic neuronal damage. We have found that ACPD is not neurotoxic itself nor does it potentiate kainate or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid neurotoxicity in cortical cultures (22). However, effects of ACPD on NMDA receptor-mediated neurotoxicity were not examined.

As a step toward a better understanding of neurotransmitter interactions in neuronal degeneration, the present study examines the effects of ACPD and carbachol, an agonist at the acetylcholine receptor, on neurotoxicity produced by brief pulse exposure to NMDA.

MATERIALS AND METHODS

Cortical Cell Culture. Primary cerebral cortical cultures were prepared from 14- to 15-day-old fetal mice generally as

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Abbreviations: NMDA, *N*-methyl-D-aspartate; EAA, excitatory amino acid; ACPD, *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; DIV, days *in vitro*; LDH, lactate dehydrogenase; PI, phosphatidylinositol; AD, Alzheimer disease; DAG, diacylglycerol; IP_3, inositol trisphosphate; PKC, protein kinase C; CPP, 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid.

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described by Choi *et al.* (23) with minor modifications (3). Dissociated cortical cells were plated in 16-mm multiwell vessels (4×10^5 cells per well) in Eagle's minimal essential medium (with Earle's salts) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM), and glucose (21 mM). After 5–10 days *in vitro* (DIV), nonneuronal cell division was inhibited by exposure to 10 μM cytosine arabinoside for 1–3 days, and the cells were shifted into a serum-free maintenance medium containing ingredients described by Brewer and Cotman (24). Subsequent medium change was carried out twice a week. For neurotoxicity experiments, only cultures of DIV 14 or greater were used, since previous studies showed that younger cortical neurons are resistant to brief glutamate exposure (23).

Exposure to EAAs. Exposure to EAAs was carried out as described (21). All agonist exposure was performed at room temperature in a Hepes-buffered salt solution with the following composition: 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM Hepes (pH 7.4 at 25°C), and 15 mM glucose. After the exposure, serum-free maintenance medium was replaced, and cultures were returned to the 5% CO_2/95% O_2 incubator until the next day, when the evaluation of neuronal degeneration was made. In most experiments, ACPD or carbachol was added to cultures along with NMDA. In some experiments, cultures were exposed for 5 min to ACPD, carbachol, or other indicated glutamate agonists in Hepes-buffered salt solution containing 50 μM 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP) and 50 μM CNQX and then exposed to NMDA for 5 min. Multiple rapid rinses were made to remove all the compounds before the NMDA exposure (calculated dilution > 2000-fold).

Quantitative Estimation of Neuronal Degeneration. Overall neuronal cell injury was estimated by examination of cultures with a phase-bright microscope (Olympus model IMT-2); damaged neurons were easily identifiable as most turned into debris (see Fig. 1*A*) and were stainable with trypan blue (0.4%, 5 min). In most experiments, neuronal cell injury was further quantitated chemically by measurement of the lactate dehydrogenase (LDH) released by damaged cells into the extracellular fluid 1 day after EAA exposure (25), using an automated microplate reader (Thermomax; Molecular Devices, Palo Alto, CA) and kinetics software. Previous control studies have demonstrated that specific LDH release is linearly proportional to the number of neurons damaged (23, 25).

Phosphatidylinositol (PI) Hydrolysis. Agonist-induced PI hydrolysis was measured as described (26). Briefly, cells were labeled for 24 hr at 37°C with 1 ml of serum-free culture medium containing 2.5 μCi (1 Ci = 37 GBq) of *myo*-[^{3}H]inositol. The cells were washed three times with 1 ml of physiological saline buffer with the following composition: 127 mM NaCl, 2 mM KCl, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 10 mM D-glucose, equilibrated with 95% O_2/5% CO_2. The cells were then preincubated for 15 min at 37°C in 500 μl of physiological saline buffer containing 10 mM LiCl, 500 nM tetrodotoxin, 50 μM CPP, and 50 μM CNQX. *trans*-ACPD was then added to 100 μM, and the incubation was continued for 10 or 30 min. The reaction was terminated by aspiration of the medium, and the cell layer in each well was extracted with 10% perchloric acid and rinsed. The precipitate was removed, and the combined supernatants were analyzed for inositol phosphate content (26).

Chemicals. All glutamate agonists and antagonists were obtained from Tocris Neuramin (Bristol, England). 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfoneamide (HA-1004) were

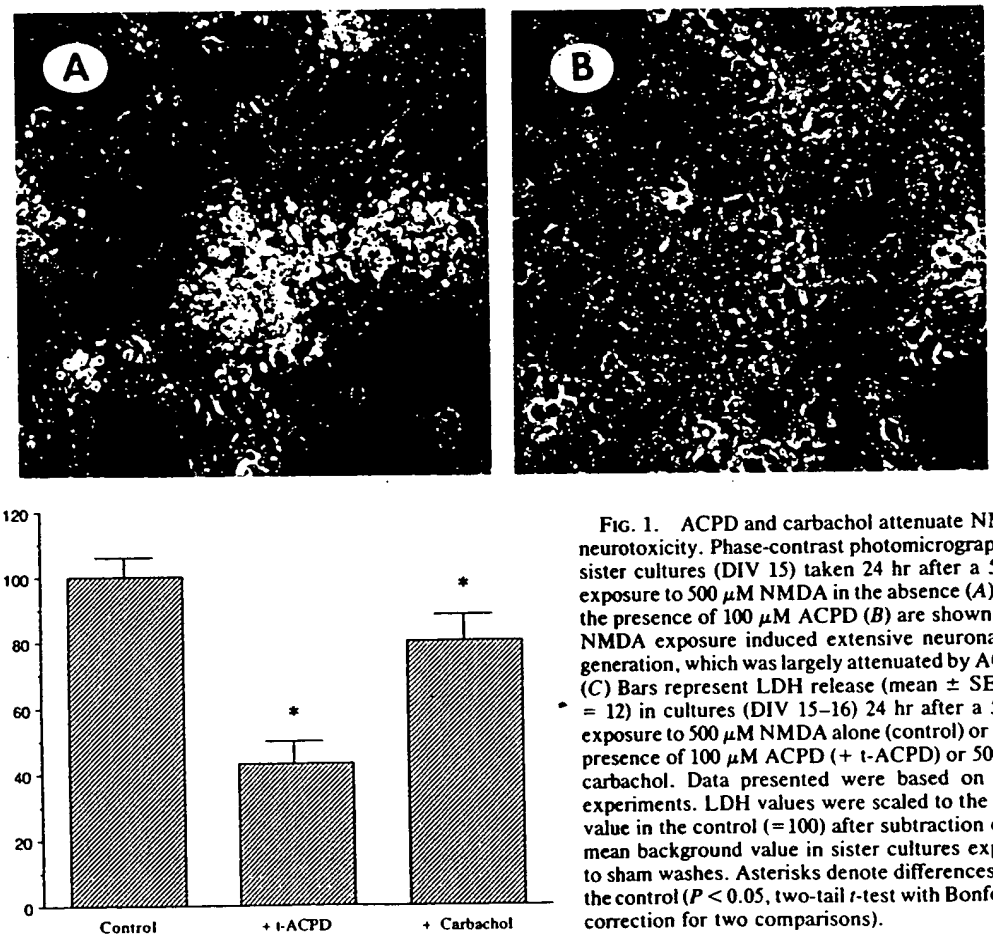

FIG. 1. ACPD and carbachol attenuate NMDA neurotoxicity. Phase-contrast photomicrographs of sister cultures (DIV 15) taken 24 hr after a 5-min exposure to 500 μM NMDA in the absence (*A*) or in the presence of 100 μM ACPD (*B*) are shown. The NMDA exposure induced extensive neuronal degeneration, which was largely attenuated by ACPD. (*C*) Bars represent LDH release (mean ± SEM; n = 12) in cultures (DIV 15–16) 24 hr after a 5-min exposure to 500 μM NMDA alone (control) or in the presence of 100 μM ACPD (+ t-ACPD) or 500 μM carbachol. Data presented were based on three experiments. LDH values were scaled to the mean value in the control (=100) after subtraction of the mean background value in sister cultures exposed to sham washes. Asterisks denote differences from the control ($P < 0.05$, two-tail t-test with Bonferroni correction for two comparisons).

obtained from Seikagaku (Rockville, MD). *myo*-[^{3}H]Inositol was obtained from Amersham. All other chemicals including carbachol were obtained from Sigma.

RESULTS

As previously reported (21), mature (≥DIV 14) cortical cultures exposed to 500 μM NMDA for 5 min showed signs of extensive neuronal degeneration over the following day (Fig. 1*A*). Immediately after the exposure, neuronal cell bodies looked swollen, neurites appeared fragmented, and, by the next day, most neurons had turned into debris. Addition of 100 μM ACPD to the NMDA exposure solution, however, often (four of six experiments) attenuated the NMDA neurotoxicity in sister cultures (Fig. 1*B*). The addition of 500 μM carbachol also reduced the NMDA neurotoxicity but to a much lesser degree. These morphological findings were confirmed by measuring LDH activity released into the bathing medium from the damaged neurons (Fig. 1*C*).

The relationship between the protective effects and the developmental stage of cultures was also examined (Fig. 2*A*). Sister cultures of different ages (DIV 14–18) were exposed to 500 μM NMDA for 5 min in the absence or presence of 100 μM ACPD or 500 μM carbachol. In 14- and 16-day-old cultures, ACPD attenuated the NMDA neurotoxicity significantly ($P < 0.05$, two-tail *t*-test with Bonferroni correction for two comparisons). However, in DIV 18 sister cultures, no protection was observed with ACPD ($P > 0.1$). In contrast, carbachol reduced the NMDA neurotoxicity by about 20% in cultures throughout the ages tested (DIV 14–18). As both ACPD and carbachol are known to induce membrane PI hydrolysis in neurons, we examined the possibility that carbachol shows less protective effect than ACPD, simply because the former is a weaker agonist for PI hydrolysis. We found no correlation between the amount of PI hydrolysis and the degree of neuroprotection; carbachol-induced PI hydrolysis was consistently larger than ACPD-induced PI hydrolysis at all ages (DIV 13–21) (Fig. 2*B*), although the protective effect was usually greater with ACPD. Furthermore, ACPD-stimulated PI hydrolysis was virtually identical at all culture ages tested, indicating that the age-dependent protection by ACPD is not entirely due to age-dependent changes in PI hydrolysis.

The dose–response relationship of ACPD required to protect against NMDA neurotoxicity was examined (Fig. 3*A*). Cultures (DIV 14–15) were exposed to 500 μM NMDA for 5 min in the presence of various concentrations of ACPD. All LDH values were represented as relative to the mean value in sister cultures exposed to NMDA alone. Some reduction in NMDA neurotoxicity was seen with 30 μM ACPD. The IC_{50} of ACPD that protected against neurotoxicity produced by a 5-min NMDA exposure was between 30 and 100 μM. To examine whether ACPD protection was dependent on NMDA concentrations, cultures (DIV 14) were exposed for 5 min to various concentrations of NMDA, either alone or in the presence of 100 μM ACPD. The protective effect of ACPD was not overcome by increasing NMDA concentrations up to 3 mM, a finding that suggests a noncompetitive mode of inhibition (Fig. 3*B*).

ACPD or carbachol also appeared to protect neurons against NMDA neurotoxicity when cultures were preexposed to these compounds. Cultures (DIV 14–15) were exposed to 100 μM ACPD or 500 μM carbachol in the presence of 50 μM CNQX and 50 μM CPP. Under this condition, neither the NMDA nor the non-NMDA receptors should be activated. Control cultures were treated the same but without ACPD or carbachol. After a 5-min exposure, multiple rinses were quickly made (<1 min) to effectively remove all of the compounds, and cultures were then exposed to 500 μM NMDA for 5 min. Preincubation in ACPD or carbachol substantially decreased NMDA neurotoxicity (Fig. 4). It should also be noted that the protective effect of carbachol was completely reversed by the coapplication of the muscarinic antagonist atropine (10 μM). The similar protective effects against NMDA neurotoxicity were also observed when cultures were preexposed for 5 min to 10 μM quisqualate in the presence of 50 μM CPP and 50 μM CNQX (Fig. 4).

Since activation of receptors linked to PI hydrolysis generates the PKC activator DAG, in addition to IP_3, we examined whether PKC or other protein kinases may mediate the protective effect of ACPD. To test this hypothesis, we used the protein kinase antagonists H-7 and HA-1004. The former is regarded as the more specific antagonist of PKC, but substantial overlap exists in concentration ranges of both H-7 and HA-1004 that act at several different protein kinases (27). A concentration of 200 μM was chosen, as similar concentrations of H-7 have previously been used and shown effective in slice preparations (28, 29). We found that both protein kinase antagonists partially reversed the protective effect of ACPD (Fig. 5), suggesting that the effect, in part, may be mediated by protein kinase(s).

DISCUSSION

The present study demonstrates that activation of the ACPD receptor substantially attenuates neurotoxicity produced by

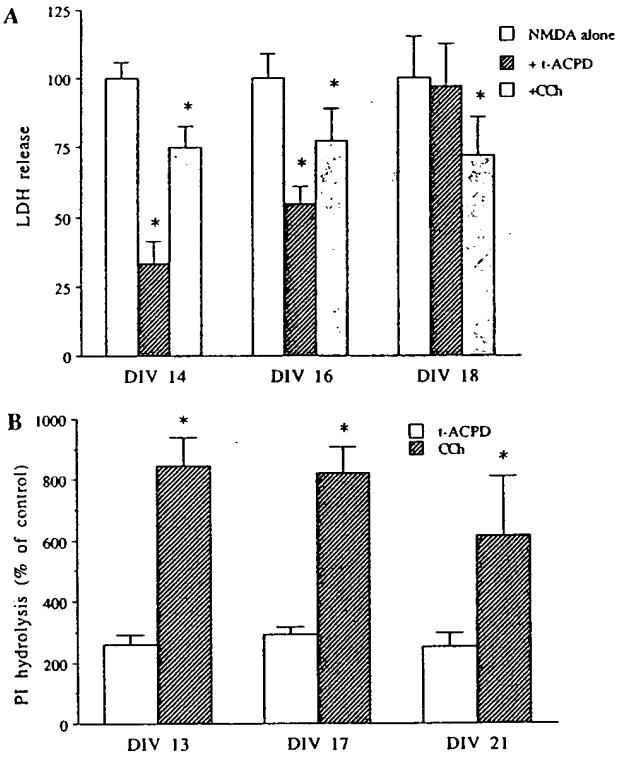

FIG. 2. (*A*) Age dependence of the ACPD effect. Data represent LDH release (mean ± SEM; $n = 7$ or 8) in cultures at different ages (DIV 14, 16, and 18) 24 hr after a 5-min exposure to 500 μM NMDA alone or in the presence of 100 μM ACPD (+t-ACPD) or 500 μM carbachol (+CCh). LDH values were scaled to the mean value in age-matched sister cultures exposed to NMDA alone (blank bars, =100). Asterisks denote differences from the control ($P < 0.05$, two-tail *t*-test with Bonferroni correction for two comparisons). (*B*) Age dependence of PI hydrolysis induced by ACPD and carbachol. Bars show levels of PI hydrolysis (percent of the control; mean ± SEM, $n = 4$) in sister cultures at different ages, after a 10-min exposure to 100 μM ACPD or 500 μM carbachol, in the presence of 50 μM CPP and 50 μM CNQX. At all ages, carbachol induced more PI hydrolysis than ACPD did (*, $P < 0.01$; two-tail *t*-test with Bonferroni correction for three comparisons). Unlike the protective effect of ACPD that decreased with culture age, PI hydrolysis by ACPD remained relatively constant throughout the culture ages tested.

a 5-min exposure to NMDA in cortical cultures. A weaker protective effect was also observed with carbachol, an agonist of the cholinergic receptor.

The protective effect of ACPD appeared to be dependent on the age of cultures in such a way that little or no protection was observed in cultures older than DIV 18. In contrast to ACPD, carbachol reduced NMDA neurotoxicity throughout the range of culture ages tested (DIV 14–18). The protective effect of carbachol observed in the present study apparently is in contrast to a previous report that acetylcholine potentiates glutamate neurotoxicity in hippocampal cultures (30). However, the pharmacology of glutamate neurotoxicity appears to be fundamentally different between the two culture systems; in the hippocampal cultures of Mattson *et al.* (31), glutamate neurotoxicity develops slowly and is largely mediated by the non-NMDA receptors, whereas in the present cortical cultures, glutamate neurotoxicity develops more rapidly (in minutes) and is largely produced by activation of the NMDA receptor (17).

As both ACPD and carbachol increase membrane PI hydrolysis in neurons (32), the protective effects may be produced by intracellular mobilization of calcium. Of note, ACPD or other agonists such as carbachol that activate PI hydrolysis often induce oscillatory increases in intracellular free calcium concentrations (22, 33, 34). It may be that the oscillatory increases in intracellular calcium render neurons more able to tolerate a subsequent calcium influx. However, a simple quantitative relationship between the level of PI hydrolysis and the degree of protection could not be found in the present study, as carbachol induced more PI hydrolysis than ACPD but provided less protection. It appears that some downstream event in the signaling cascade is not responsive to the activation of PI hydrolysis at later developmental periods.

The concentration range of ACPD that attenuates the NMDA neurotoxicity (30–300 μM) is consistent with the range in which ACPD increases PI hydrolysis in hippocampal cultures. Interestingly, but not unexpectedly, the mode of protection appears

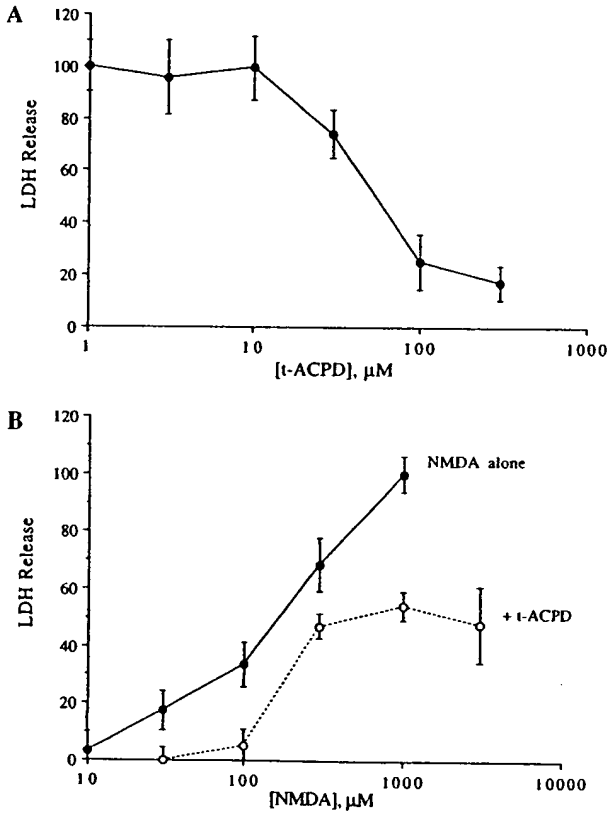

FIG. 3. (*A*) Concentration–protection relationship of ACPD. Data represent LDH release (mean ± SEM; n = 4–12) in cultures (DIV 14–15) 24 hr after a 5-min exposure to 500 μM NMDA in the presence of various concentrations of ACPD (t-ACPD). The protective effect of ACPD was observed at concentrations between 30 and 300 μM. (*B*) Noncompetitive mode of protection by ACPD against the NMDA neurotoxicity. Sister cultures (DIV 14) were exposed for 5 min to various concentrations of NMDA alone or in the presence of 100 μM ACPD. Data represent LDH release (mean ± SEM; n = 4) in the bathing media 24 hr after the exposure. The protective effect of ACPD remained unchanged with NMDA concentrations between 300 and 3000 μM. LDH values were scaled to the mean value in sister cultures exposed to 1 mM NMDA (=100) after subtraction of mean background values.

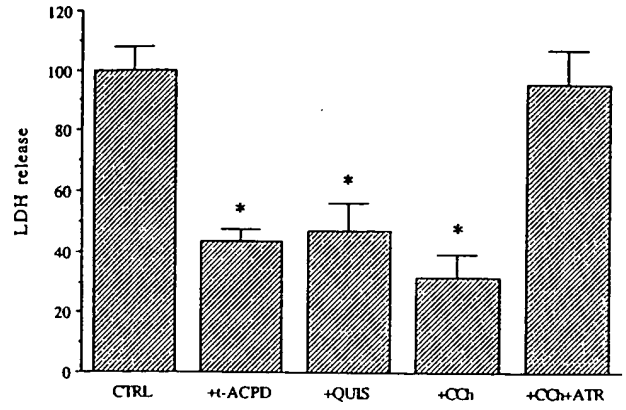

FIG. 4. Preincubation in ACPD or carbachol also reduces subsequent NMDA neurotoxicity in the presence of CNQX and CPP. Cultures (DIV 14–15) were exposed for 5 min to 100 μM ACPD (+t-ACPD), 10 μM quisqualate (+QUIS), or 500 μM carbachol (+CCh) [alone or with 10 μM atropine (+ATR)] in the presence of 50 μM CPP and 50 μM CNQX to block both NMDA and non-NMDA receptors. Immediately after a 5-min preexposure, cultures were washed several times to remove preexposure chemicals and exposed to 500 μM NMDA for 5 min. Control cultures were preexposed to CPP and CNQX alone and subsequently exposed to NMDA (CTRL). Bars represent LDH release (mean ± SEM) 24 hr after the NMDA exposure. Asterisks denote differences from the control ($P < 0.05$, two-tail t-test with Bonferroni correction for five comparisons).

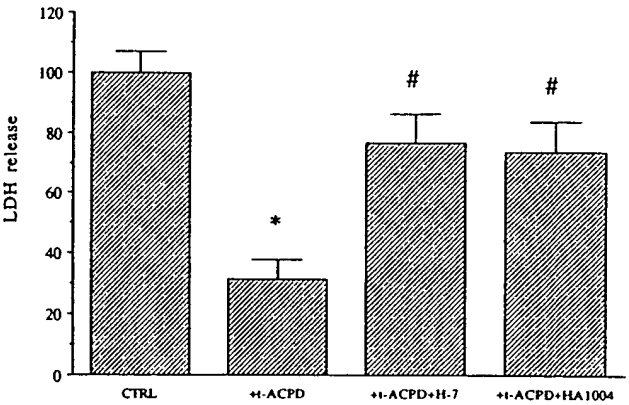

FIG. 5. Antagonists of protein kinases partially reverse the protective effect of ACPD on the NMDA neurotoxicity. As shown above, cultures (DIV 14) exposed to ACPD and NMDA (+t-ACPD) showed less neuronal degeneration and less LDH release than cultures exposed to NMDA alone (CTRL). However, the protective effect of ACPD appears to be largely diminished when H-7 (200 μM) or HA-1004 (200 μM) was applied to cultures 5 min before the exposure; antagonists were available throughout the NMDA exposure. Bars depict mean LDH release (± SEM; n = 4). An asterisk denotes a difference from the control; a pound sign denotes a difference from both the control and the cultures exposed to ACPD and NMDA ($P < 0.05$, two-tail t-test).

to be noncompetitive. Even at saturating concentrations of NMDA, ACPD was effective in protecting neurons.

Of interest, the protective effect produced by ACPD or carbachol was seen even when one of these compounds was applied before the NMDA exposure, suggesting that the cellular changes induced by these agonists may last at least a few minutes following the washout. Also, the fact that the protection by ACPD or quisqualate was observed even in the presence of CPP and CNQX further suggests that these effects are not likely produced by indirect activation of the ionotropic glutamate receptors.

In addition to IP_3, DAG, an activator of PKC, would be produced by ACPD or carbachol. The partial reversal of the protection by H-7 and HA-1004 suggests that protein kinases may be involved, in part, in the protective cascade, although it is difficult to determine whether PKC alone is specifically involved, due to the fact that the actual concentrations achieved in the neuronal membrane are unknown. At high concentrations, biochemical assays have shown that H-7 and HA-1004 can block multiple protein kinases (27). More detailed pharmacological studies are needed to identify the kinases or other events involved.

It is possible that negative modulatory interactions exist between the ACPD receptor and the NMDA receptor. In fact, our previous study has shown that activation of NMDA receptors inhibits ACPD-induced PI hydrolysis in hippocampal slices (26). The present study suggests that the reverse may also exist—i.e., that ACPD receptor activation may reduce responses mediated by the NMDA receptor.

During development, the appearance of the ACPD receptor exhibits a transient peak that coincides with synaptogenesis. For example, PI hydrolysis by activation of the ACPD receptor peaks during 6–12 days after birth in rat hippocampal slices (35). The functional significance of this transient increase is currently unknown, but it has been suggested to play a role in synaptogenesis (35, 36). The NMDA receptor shows similar up-regulation during development (37). Consistent with the transient up-regulation of the NMDA receptor, it has been reported that in young animals NMDA produces more excitotoxic neuronal damage compared to that in adult animals (38). This NMDA receptor up-regulation may normally serve an important function, such as mediating developmental synaptic plasticity in visual cortex (39, 40). The present finding that ACPD attenuates NMDA neurotoxicity in cortical cultures in an age-dependent manner suggests that the transient increase in ACPD receptor *in vivo* also might serve as a necessary protective mechanism against brief, intensive NMDA receptor activation, necessary for normal synaptic plasticity during central nervous system development.

In summary, the present study demonstrates that neurotransmitter interactions may be able to affect the expression of excitotoxic neuronal damage. It may prove clinically important to understand all the factors that can alter excitotoxicity, in light of substantial evidence linking this type of neurotoxicity to a wide variety of neurodegenerative conditions (1, 2). For example, in AD, a marked reduction in cholinergic neurotransmission is probably one of the early abnormalities (4, 41). In addition to the direct functional consequences, the present data suggest that the cholinergic deficit may also subsequently increase the risk of target neurons to excitotoxic injury. Since it is known that central neurons containing neurotransmitters such as somatostatin (5, 6), serotonin (42), and norepinephrine (43) are also affected in neurodegenerative diseases, it may be interesting to see if these neurotransmitters can modulate excitotoxicity as well.

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Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity

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ABSTRACT **Defects in neurotransmitter glutamate transport may be an important component of chronic neurotoxicity in diseases such as amyotrophic lateral sclerosis. There are no reliable models of slow glutamate neurotoxicity. Most previous *in vitro* systems have studied the rapid neurotoxic effects of direct-acting glutamate agonists. Therefore, we developed a model of slow toxicity in cultured organotypic spinal cord slices. The model was based on selective inhibition of glutamate transport, which continuously raised the concentration of glutamate in the culture medium. This resulted in the slow degeneration of motor neurons over several weeks. Motor neuron toxicity was selectively prevented by non-*N*-methyl-D-aspartate glutamate receptor antagonists and glutamate synthesis or release inhibitors but not by *N*-methyl-D-aspartate receptor antagonists. Thus, selective inhibition of glutamate transport produces a model of clinically relevant slow neurotoxicity and appears to be mediated by the action of non-*N*-methyl-D-aspartate receptors. This data supports the hypothesis that the slow loss of motor neurons in amyotrophic lateral sclerosis could be due, in part, to defective glutamate transport.**

Amyotrophic lateral sclerosis (ALS) is characterized clinically by progressive weakness and wasting of muscles caused by a slow loss of large and small neurons, predominantly motor neurons, in the ventral spinal cord, brainstem, and motor cortex. Previous studies (1, 2), including those showing increased cerebrospinal fluid glutamate concentrations, have demonstrated that the disease is associated with abnormal glutamate metabolism. Recently, a loss of high-affinity glutamate transport was identified in certain brain regions and spinal cord of patients with ALS (3). These results suggested that the defect in glutamate transport could be responsible for sustained elevations in extracellular glutamate, which might be injurious to neurons. Under normal conditions, glutamate is rapidly cleared from the synaptic cleft by high-affinity high-capacity transport into both presynaptic terminals and nearby astrocytes (4). The glutamate carrier can be specifically and potently inhibited by both threohydroxyaspartate (THA) and pyrrolidine dicarboxylic acid (PDC) (5, 6). We developed an *in vitro* model of defective glutamate transport, by chronically blocking the glutamate carrier, to test the hypothesis that inefficient glutamate transport would result in elevation of extracellular glutamate, and if sustained, might produce slowly developing neurotoxicity. We used organotypic spinal cord cultures in this model, cultures that have the advantage of long-term survival with partially preserved synaptic connections, to mimic as closely as possible the transport defect that is known to occur in ALS (3).

MATERIALS AND METHODS

Experimental Design. In all experiments, cultures were allowed an 8-day recovery period after initial preparation, prior to the addition of drugs. To produce chronic toxicity, long-term inhibition of glutamate uptake was effected by incubating slices with culture medium containing various concentrations of THA or PDC (5, 6). At very high concentrations (>1 mM), THA can have weak actions at the *N*-methyl-D-aspartate (NMDA) receptor, a property not shared by PDC (5). Transport inhibitors were maintained in cultures by replenishing them at each change in culture medium. In all experiments, potentially neuroprotective drugs were added repeatedly, either in the presence or absence of a glutamate transport inhibitor, again beginning after 8 days in culture. Motor-neuron toxicity was monitored by two methods: (*i*) biochemical analysis of tissue choline acetyltransferase activity (ChAT) and (*ii*) microscopic morphology. ChAT activity is largely restricted to ventral motor neurons in rat lumbar spinal cord, and assays of ChAT activity have been used as a reliable marker for motor neurons (7–9). Motor neurons were also visualized in organotypic cultures by histological analysis of stained semithin plastic sections and by immunohistochemistry.

Organotypic Spinal Cord Cultures. Organotypic spinal cord cultures were prepared using lumbar spinal cord slices from 8-day-old rat pups. Neonatal rat pups were decapitated, and the spinal cords were rapidly harvested and cultured by a modification of described methods (10, 11). Lumbar spinal cords were collected under sterile conditions and sectioned transversely at 350-μm intervals with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, U.K.). Sections were then transferred to sterile Gey's balanced salt solution (GIBCO) containing glucose (6.4 mg/ml) and gently separated at room temperature. Slices were carefully placed on the surface of 30-mm Millipore Millicell-CM porous (0.4 μm) membranes (five slices per membrane). Such tissue grows optimally at an air/fluid interface, so it was important to remove any excess medium on the membrane surface around slices. The membranes were placed in 35-mm culture wells (Nunc) containing 1 ml of incubation medium [50% (vol/vol) minimal essential medium–25 mM Hepes/25% (vol/vol) heat-inactivated horse serum/25% (vol/vol) Hanks' balanced salt solution (GIBCO) supplemented with D-glucose (25.6 mg/ml) and glutamine (2 mM), at a final pH of 7.2]. Initial studies using pH 7.4 or 7.8 revealed similar results. Antibiotic and antifungal agents were not used. Cultures were incubated at 37°C in a 5% CO_2/95% air humidified environment (Forma Scientific, Marietta, OH). Culture medium, along with any added pharmacological agents, was changed twice weekly. By using this technique, >95% of the explants can be maintained in culture for >3 months with excellent organotypic cellular organization.

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Abbreviations: ALS, amyotrophic lateral sclerosis; ChAT, choline acetyltransferase; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; MSO, methionine sulfoximine; NMDA, *N*-methyl-D-aspartate; PDC, pyrrolidine dicarboxylic acid; THA, threohydroxyaspartate; TTX, tetrodotoxin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid.

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Culture Morphology and Immunohistochemistry. Motor-neuron survival was evaluated by two procedures. (*i*) Cultures fixed in 4% (vol/vol) glutaraldehyde and embedded in plastic were sectioned at 2 μm continuously from top-to-bottom. Motor neurons, operationally defined as cells in the ventral horn region >30 μm in diameter, were then counted in every 20th section. (*ii*) ChAT-positive motor neurons, in the bilateral ventral gray region of whole cultures, were identified and counted immunohistochemically using the peroxidase-antiperoxidase double-bridge method (12) with ChAT monoclonal antibody (Boehringer Mannheim) at a 1:100 dilution and developed using diaminobenzidine. Control cultures were incubated without the primary antibody.

Biochemical Assays. To determine ChAT activity, the spinal cord tissue in each dish (five slices) was pooled and frozen (−80°C) until assay. Each culture well represented one time point or drug concentration. ChAT activity was measured radiometrically (13) by using [^{3}H]acetyl-coenzyme A (Amersham). Somatostatin was measured by RIA (Peninsula Laboratories). Protein content of tissue homogenates was determined by a Coomassie protein assay kit (Pierce).

Glutamate Determinations. Glutamate was assayed in samples (20 μl) of cell culture medium at weekly intervals after initiation of THA treatment. Glutamate levels were determined fluorometrically in nonacidified samples by an automated HPLC method (14) that can detect amounts as low as 0.1 μmol/liter.

Drugs. THA, methionine sulfoximine (MSO), and tetrodotoxin (TTX) were from Sigma, and PDC was from Tocris Neuramin (Bristol, England). Purity of THA was confirmed by reverse-phase HPLC (Waters) as described (14). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), NMDA, and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) were obtained from Research Biochemicals (Natick, MA). 1-(4-Aminophenyl)-4-methyl-7,8-methylene-dioxy-5*H*-2,3-benzodiazepine hydrochloride (GYKI-52466) was generously supplied by Istvàn Tarnawa (Institute for Drug Research, Budapest).

RESULTS

Spinal cord cultures were reliably maintained for as long as 3 months and had well-preserved organotypic morphology. Dorsal horn neurons could be identified by the typical high density of tightly packed small cells (Fig. 1*A*). Somatic motor neurons were readily identifiable by their large size and distinctive grouping in the ventral lateral region of the explant culture (Fig. 1*A*). Immunohistochemical stains for the motor-neuron-specific enzyme ChAT identified clumps of motor neurons in the lateral ventral horn region (Fig. 1*B*).

ChAT activity initially dropped precipitously for the first several days after culturing but gradually returned to baseline values (Fig. 2), levels that were also comparable to age-matched uncultured lumbar spinal cord tissue (data not shown). The initial drop in enzyme activity is a typical response of motor neurons to axotomy, not a loss of cells; the return of ChAT activity reflects the recovery from this initial injury (7–9). This conclusion was supported by morphological analysis of the ventral motor neurons that were normal in appearance after a period of mild reversible chromatolytic changes limited to the first 3 weeks in culture (Fig. 3*A*). Furthermore, counts of ChAT-positive neurons revealed a stable population of ventral motor neurons throughout the period of the culture (Fig. 4*A*).

Chronic inhibition of glutamate transport with THA produced a dose-dependent persistent elevation of glutamate levels in the culture medium (Fig. 5). Inhibition of glutamate transport by THA also produced a slow loss of the motor neuron marker ChAT, and the rate at which toxicity occurred was dose-dependent (Fig. 2). High concentrations of THA (500 μM), produced motor-neuron toxicity after 1 week; at lower concentrations of THA (100 μM), toxicity began after 2–3 weeks. Lower concentrations of THA (25 μM) caused only slight decreases in ChAT after 45 days, and 10 μM THA was not toxic up to 72 days in culture. Similar results were found using comparable concentrations of PDC (Table 1).

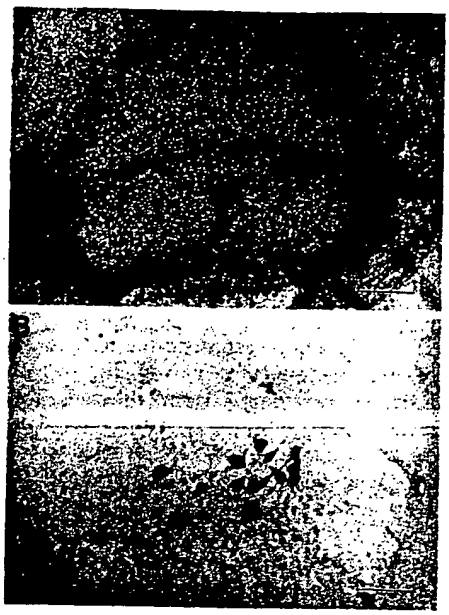

FIG. 1. Organotypic cultures of rat lumbar spinal cords. (*A*) Five-week-old culture stained with cresyl violet. Typical morphology of dorsal horn (DH), ventral horn (VH), and central canal (arrow) are visible. Outer edge of culture is surrounded by glial scar from degenerated white matter tracts. (Bar = 1 mm.) (*B*) ChAT-positive motor neurons in ventral horn in 2-week-old culture. (Bar = 100 μm.)

To test whether delayed toxicity was dependent on the developmental stage of the tissue, organotypic cultures were also maintained for 30 and 50 days before the addition of transport inhibitors. We still observed delayed toxicity with

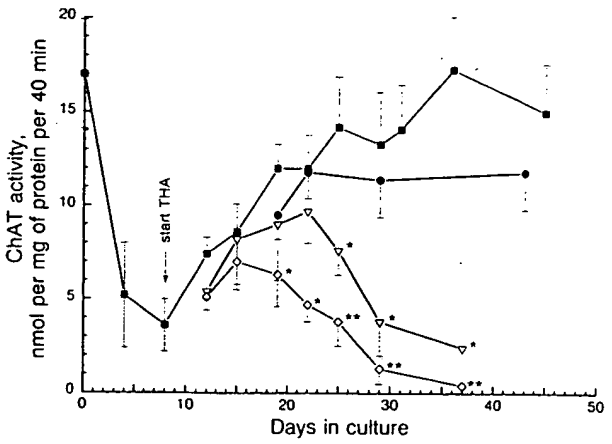

FIG. 2. ChAT activity in spinal cord organotypic cultures as a marker for motor-neuron toxicity in the presence of THA. Cultures were prepared from lumbar spinal cords, and after 8 days in culture, THA at 25 (●), 100 (▽), or 500 (◇) μM was added to the culture medium and was maintained in all subsequent medium changes. At selected times, ChAT activity was measured in homogenates from control (■) or THA-treated cultured spinal cord tissue explants. Each time point represents the mean ± SEM of 6–21 replicate culture wells, each containing five slices. Statistical significance (independent *t*-test) of THA treatment vs. control: *, $P < 0.05$; **, $P < 0.01$.

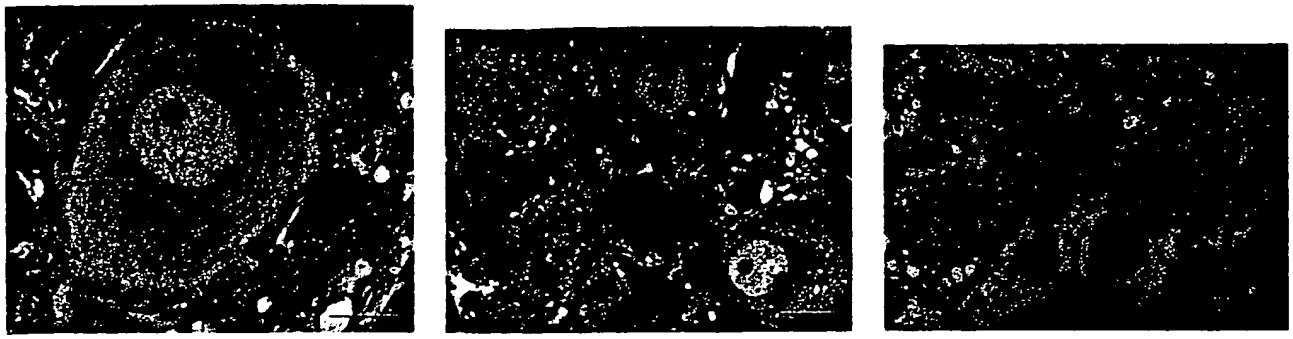

FIG. 3. Neurotoxicity produced by chronic inhibition of glutamate uptake in organotypic cultures of lumbar spinal cord. (*A*) Typical motor neuron in 22-day-old control cultures. (*B*) Mild vacuolar degeneration of motor neurons (arrow) after chronic (1 week) treatment with a low dose of THA (100 μM). Astrocytes and oligodendrocytes appeared intact. (*C*) Severe vacuolar degeneration of motor neurons after chronic treatment (14 days) with 500 μM THA. (Bar = 10 μm.) Arrows indicate vacuoles.

a similar time course in these aged cultures using both 100 μM and 500 μM THA. PDC or THA did not cause acute motor-neuron toxicity (i.e., within 1–4 days).

Chronic treatment of cultures with THA (100 μM and 500 μM) produced a vacuolar degeneration initially limited to motor neurons. After 1 week of treatment with low concentrations of THA (100 μM), motor neurons frequently contained many cytoplasmic vacuoles (Fig. 3*B*), which were not seen in age-matched control cultures. After 14 days of treatment with high concentrations of THA, there was severe vacuolar degeneration of motor neurons (Fig. 3*C*). However, at the same time motor neurons demonstrated vacuolar degeneration, surrounding astrocytes, oligodendrocytes, and small neurons appeared undamaged in the ventral horn (Fig. 3*C*) and dorsal horn region. Severe degeneration of the entire tissue was not seen until 28 days with THA 500 μM and 35 days in cultures treated with THA 100 μM. The vacuolar nature of the earliest morphological changes and the sparing of nonneuronal cells are typical of glutamate-induced neurotoxicity (15). Counts of ChAT-positive cells (Fig. 4*A*) and counts of large ventral horn cells (>30 μm in diameter) in the plastic thick sections (Fig. 4*B*) also confirmed the loss of motor neurons after 20–25 days of treatment with 100 μM THA.

To obtain biochemical evidence for what appeared morphologically to be a relatively selective toxicity, we measured spinal cord somatostatin levels. Somatostatin is largely localized to cell bodies in the dorsal gray matter of the spinal cord (16). Somatostatin and ChAT levels were unchanged from control levels in 22-day-old cultures treated with 100 μM THA. By 29 days in culture, when 100 μM THA caused

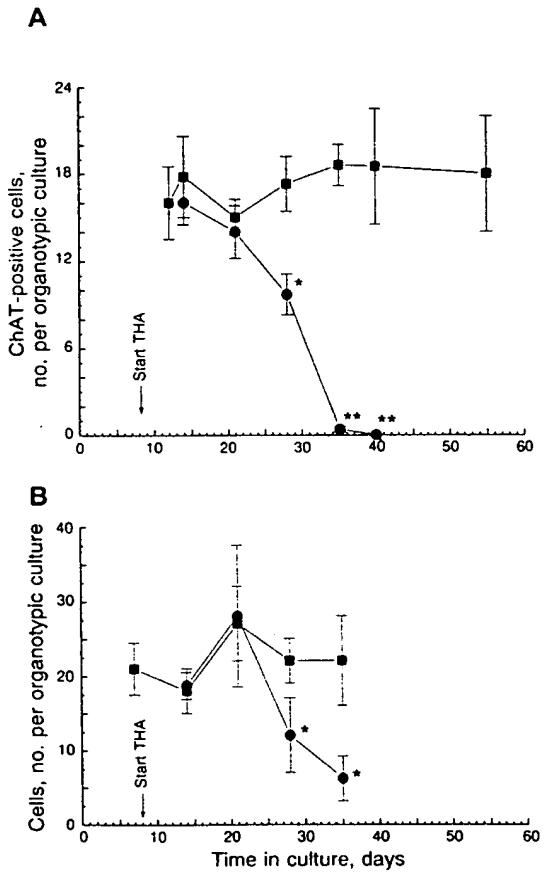

FIG. 4. (*A*) Immunohistochemically defined motor neurons in spinal cords during the first 10 weeks of organotypic culture. All ChAT-positive cells in the bilateral ventral horn region of spinal cord organotypic cultures were counted. Each point represents the cell count (mean ± SEM) from 20–50 explants. (*B*) Counts of spinal cord motor neurons >30 μm in diameter in serial sections of plastic-embedded cultures. Each point represents the cell count (mean ± SEM) from two to four explants. Statistically significant differences of THA (100 μM) treatment (●) vs. control (■) (independent *t* test): *, $P < 0.05$; **, $P < 0.01$.

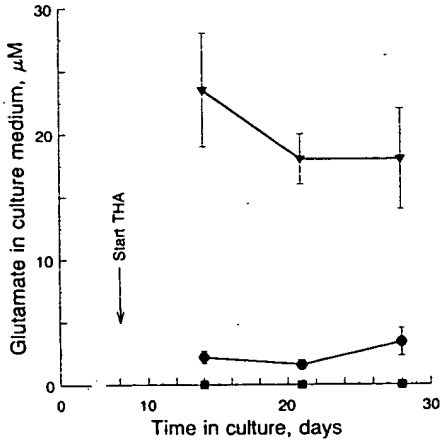

FIG. 5. Sustained elevation of glutamate levels in culture medium after chronic exposure to THA. Organotypic cultures were maintained for 8 days, and then THA was added to 100 μM (●) or 500 μM (▼). Glutamate was measured in aliquots of medium before it was changed. Prepared medium contains 8.1 ± 0.9 μM glutamate (mean ± SEM), but glutamate in medium from control cultures (■) was below the lower limits of detection because of normal high-affinity glutamate transport in neural tissue. Each point represents the mean ± SEM of four to eight culture wells.

Table 1. ChAT activity in spinal cord organotypic cultures as a marker for motor-neuron toxicity in the presence of PDC

Time in culture, days	% of control ChAT activity	
	100 μM PDC	400 μM PDC
15	100 ± 7.8	67 ± 5.3*
22	69 ± 5*	18 ± 6.2**
29	33.5 ± 8.5*	0**

After 8 days in culture, PDC was added to the culture medium to 100 or 400 μM and was maintained in all subsequent biweekly changes of medium. Data are expressed as percent of control ChAT activity (mean ± SEM). Each mean represents four to eight replicate culture wells, each containing five slices. Statistical significance (independent *t* test) of PDC treatment vs. control: *, $P < 0.05$; **, $P < 0.01$.

a 70% reduction of ChAT ($P < 0.01$), somatostatin levels were not significantly changed from control levels.

To determine whether chronic toxicity was mediated by a specific glutamate receptor subtype, cultures were incubated concomitantly with the transport inhibitor and various glutamate receptor antagonists, including: the noncompetitive NMDA receptor antagonist MK-801, the competitive NMDA antagonist CPP, the noncompetitive non-NMDA receptor antagonist GYKI-52466, or the competitive non-NMDA antagonist CNQX (17, 18). Both GYKI-52466 and CNQX provided nearly complete protection of motor neurons from glutamate-mediated toxicity at 22, 25, and 29 days in culture. MK-801 and CPP were unable to protect motor neurons from toxicity mediated by long-term inhibition of glutamate uptake (Fig. 6*A*).

When the potent agonists NMDA or AMPA (a selective non-NMDA agonist) were directly and repeatedly added to culture medium over a range of concentrations, only AMPA was capable of producing motor-neuron toxicity (Fig. 6*B*).

If the increased extracellular glutamate, due to inhibition of glutamate transport by THA, was the cause of slowly progressive neurotoxicity, then interruption of the cellular release of glutamate should protect against this toxicity. MSO irreversibly inhibits glutamine synthetase, a synthetic enzyme for neurotransmitter glutamate (4), thereby depleting presynaptic pools of neurotransmitter glutamate (19, 20). When spinal cord cultures were incubated with 1 mM MSO, the culture medium glutamate levels were not elevated and the motor neurons were no longer susceptible to the toxic effects of high concentrations of THA (Fig. 6*A*).

TTX can block activity-dependent release of neurotransmitter and can be neuroprotective (21). When TTX (1 or 10 μM) was continuously added to cultures, medium glutamate levels were not increased and motor neurons were no longer susceptible to the toxic effects of THA (Fig. 6*A*).

DISCUSSION

Organotypic cultures prepared from postnatal rat lumbar spinal cord provide a unique method to chronically study motor-neuron properties. We found that the cultures could be maintained with appropriate morphological features, including a stable population of ventral motor neurons, for at least 3 months. By exposing cultures to specific glutamate transport inhibitors, we have developed a reproducible model that mimics a feature of sporadic ALS, that is, the loss of glutamate transport. Using this paradigm, we have discovered two significant characteristics of glutamate toxicity in spinal cord neurons, (*i*) chronic exposure to excessive extracellular glutamate can lead to the slow loss of motor

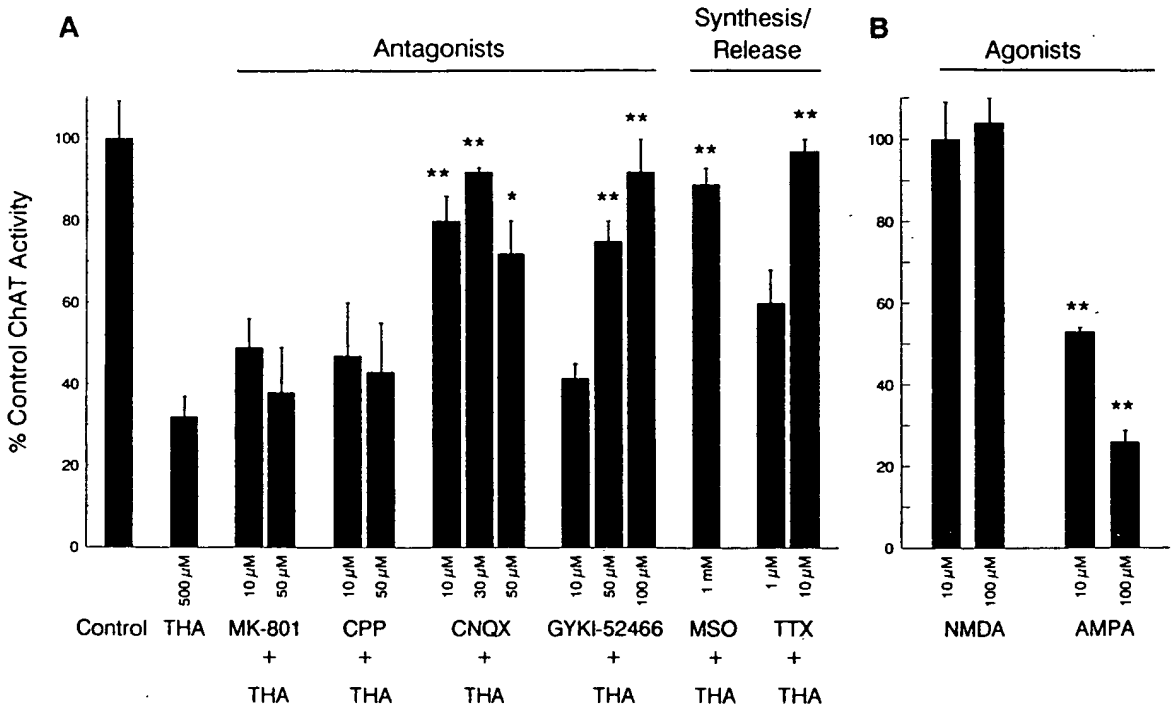

FIG. 6. (*A*) Effects of selective disruption of glutamatergic neurotransmission on THA-mediated motor-neuron toxicity. Spinal cord organotypic cultures were incubated with 500 μM THA and treated continuously with various possible neuroprotective drugs as indicated. Control cultures also were tested with the putative protective drug alone without THA. There were no significant effects of the neuroprotective agents when studied alone. Significance of a drug's protective effect was tested by the independent *t* test comparing drug plus THA vs. THA alone. (*B*) Toxic effects of sustained exposure of spinal cord neurons to glutamate receptor agonists. NMDA or AMPA was added to organotypic cultures at the indicated concentrations and compared with the effect of THA. Results are presented as a percentage of ChAT activity in age-matched untreated control cultures. For all experiments, culture medium, along with added pharmacological agents, was changed twice weekly. At 25 days in culture, spinal cord tissue was assayed for ChAT activity. (*A* and *B*) Each time point represents the mean ± SEM from 8–20 replicate culture wells, each containing five slices. Statistical significance (independent *t* test): *, $P < 0.05$; **, $P < 0.01$.

neurons (i.e., over weeks to months) and (*ii*) motor neurons are uniquely sensitive to non-NMDA but not NMDA receptor-mediated toxicity.

THA or PDC produced motor-neuron toxicity in a dose-dependent fashion by blocking glutamate uptake, which leads to a chronic accumulation of extracellular glutamate. Transport blockade, the mechanism by which THA produced toxicity, was indirectly verified in the experiments employing drugs (MSO and TTX) that could interfere with presynaptic release of glutamate. The neuroprotection afforded by both these agents verifies the need for glutamate synthesis and release in THA-mediated neurotoxicity.

Motor-neuron loss in this model was confirmed by three methods: biochemical measurements of ChAT, counts of large ventral horn cells in serial plastic sections of organotypic cultures, and counts of ChAT-immunoreactive large ventral horn cells. The loss of ChAT activity in homogenates corresponded closely with both the loss of large ventral horn neurons and the loss of ChAT-positive cells. Thus, in this paradigm, chronic toxicity represents true loss of motor neurons rather than just down regulation of ChAT activity.

Morphologic evaluation of the cultures revealed that motor neurons were preferentially altered first in this model, as shown by the vacuolar changes seen even before the biochemical indices had changed. The disparate effect of THA on ChAT and somatostatin also suggests an initial selective vulnerability to THA-induced glutamate toxicity among motor neurons rather than dorsal horn cells.

Glutamate toxicity can be mediated by both NMDA and non-NMDA glutamate receptor subtypes in acute models of cell death (22). Motor neurons appear to possess both NMDA and non-NMDA receptors (23–25); however, the relative preponderance of these subtypes on motor neurons is not known. The postnatal motor neurons used in this culture were particularly susceptible to glutamate toxicity mediated through the non-NMDA receptors, as shown by the fact that non-NMDA antagonists could effectively prevent the uptake-mediated chronic neurotoxicity and that AMPA was a potent motor-neuron toxin. However, NMDA antagonists were not neuroprotective, and NMDA itself was not toxic to motor neurons. Acute non-NMDA-mediated toxicity affecting motor neurons has been suggested by studies of chicken spinal cord *in vitro* (26) and neonatal rats *in vivo* (27). In contrast, our model clearly demonstrates selective non-NMDA-mediated slow neurotoxicity.

The cellular mechanisms by which cells slowly degenerate after chronic inhibition of glutamate uptake are not known, although it appears to be mediated via non-NMDA receptors. Could the cellular localization of non-NMDA receptors or the pattern of non-NMDA receptor subunit combinations determine the selective loss of motor neurons in ALS? Interestingly, genetic analysis of the autosomal dominant form of ALS, which is quite similar phenotypically to the more prevalent sporadic ALS, has revealed a mutation of the Cu/Zn superoxide dismutase gene (28). Superoxide dismutase converts superoxide anions into H_2O_2 and O_2. Excess oxygen radicals have been postulated to participate in the injury associated with glutamate acting at non-NMDA receptors (29). Perhaps a selective localization of glutamate receptor subtypes and abnormal cellular mechanisms for detoxification of free radicals are responsible for the slow death of motor neurons in our culture and in familial ALS (30). Abnormalities in other cellular cascades (e.g., calcium homeostasis, inositol phospholipid metabolism, and age-related mitochondrial gene defects) could also act in series with ongoing glutamate-mediated toxicity to produce the selective chronic motor-neuron degeneration recognized as the phenotype of ALS. The organotypic spinal cord culture model provides a convenient way to test some of these hypotheses.

Based on this model of chronic neurotoxicity, the selective slow loss of motor neurons observed in ALS could be due, in part, to the loss of glial or neuronal glutamate transport, with concomitant persistent exposure of neurons to elevated synaptic glutamate. The protection from chronic motor-neuron toxicity provided by non-NMDA antagonists or inhibitors of presynaptic release may have important therapeutic implications.

We thank J. Vornov for helpful discussions and for providing initial training on organotypic methodology. R. Chang helped in the initial development of our organotypic culture technique. M. Lehar provided technical assistance. This work was supported by National Institutes of Health Grants NS30086 and KO8NSO1355, the Jay Slotkin Fund for Neuromuscular Research, and the Muscular Dystrophy Association.

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Exhibit S (10/644,645)

(1S,3R)-1-AMINOCYCLOPENTANE-1,3-DICARBOXYLIC ACID ATTENUATES *N*-METHYL-D-ASPARTATE-INDUCED NEURONAL CELL DEATH IN CORTICAL CULTURES VIA A REDUCTION IN DELAYED Ca^{2+} ACCUMULATION

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(Accepted 3 August 1993)

Summary—The effects of (1S,3R)-ACPD, a selective metabotropic glutamate receptor agonist, on NMDA-induced $^{45}Ca^{2+}$ accumulation and delayed neuronal cell death were determined using primary cerebrocortical cultures. Exposure to (1S,3R)-ACPD alone, although causing small increases in $^{45}Ca^{2+}$ accumulation, was not neurotoxic. The presence of (1S,3R)-ACPD during exposure to NMDA attenuated the resulting sustained accumulation of $^{45}Ca^{2+}$ and delayed neuronal cell death. Reductions in sustained Ca^{2+} accumulation were associated both with Ca^{2+} efflux, in the absence of cell death, and inhibition of delayed intracellular Ca^{2+} accumulation. The protective effects of (1S,3R)-ACPD on NMDA-induced cell death were inhibited by pretreatment of cultures with pertussis toxin. These results suggest that activation of metabotropic glutamate receptors may stimulate intracellular processes capable of limiting sustained elevations in intracellular calcium and the resulting excitotoxic neuronal damage.

Key words—glutamate metabotropic receptors; neurotoxicity; excitotoxicity; phosphoinositide.

The excitatory amino acid glutamate is thought to be involved in neuronal cell death associated with a range of neurodegenerative and hypoxic-ischemic cerebral disorders [see Meldrum and Garthwaite (1990); Farooqui and Horrocks (1991)]. Glutamate activates two major groups of receptors. Glutamate ionotropic receptors are divided into three main subtypes including *N*-methyl-D-aspartate (NMDA), kainate and alpha-amino-hyroxy-5-methyl-4-isoxazolepropionoic acid (AMPA) receptors (Monaghan, Bridges, and Cotman, 1989; Watkins, Krogsgaard-Larsen and Honore, 1990), the stimulation of which directly activates cation channels permeable to Na^{2+} or Ca^{2+}. Glutamate acting via metabotropic, or (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD] sensitive receptors, stimulates a variety of intracellular second messenger responses [see Schoepp, Bockaert and Sladeczek, (1990a)]. Recently six subtypes of metabotropic glutamate receptor have been identified using molecular cloning techniques. Functional expression of metabotropic glutamate receptor subtypes has revealed that they may be differentially linked to activation of phospholipase C or to inhibition of cAMP accumulation [see Nakanishi (1992)].

The cytotoxicity of glutamate is linked to increases in intracellular Ca^{2+} (Choi, 1988), and, although blockade of ionotropic receptors results in the inhibition of glutamate-induced neuronal injury, the role played by metabotropic receptor activation is unclear. In cortical cultures *trans*-ACPD, a potent and selective agonist at metabotropic glutamate receptors (see Schoepp *et al.*, 1990a), is not in itself neurotoxic (Koh, Palmer, Lin and Cotman, 1991a), and when applied together with NMDA, *trans*-ACPD attenuates the resulting neuronal cell death (Koh, Palmer and Cotman, 1991b). Likewise, (1S,3R)-ACPD, the active isomer of *trans*-ACPD, reduces the excitotoxic effects of intraoccular injection of NMDA on the retina (Siliprandi, Lipartiti, Fadda, Sautter and Manev, 1992). The present study investigated further the role played by metabotropic receptors in glutamate-induced neuronal cell death in primary cerebrocortical cultures. Potential mechanisms involved in the neuroprotective effects of metabotropic receptor activation, with particular emphasis on intracellular Ca^{2+} levels, were examined using (1S,3R)-ACPD.

METHODS

Primary cerebrocortical cultures were prepared as described previously (Marcoux, Probert and Weber, 1990). In brief, cortical hemispheres were sectioned from foetal rat brain and dissociated in Hank's Balanced Salt Solution (HBSS) containing 0.1% trypsin. Cells were triturated and suspended in Dulbecco's Modified Eagles Medium (DME) and

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Ham's Nutrient Mixture F12 (F-12) supplemented with 10% horse serum and 6% foetal calf serum, and plated out at a density of 200,000 cells/cm^2. Additions of 5-fluoro-deoxyuridine (15 μg ml^{-1}) and uridine (35 μg ml^{-1}) were made after four days incubation to inhibit further division of non-neuronal cells (Ranssom, Neale, Henkart, Bullock and Nelson, 1977). Cultures were fed as necessary using DME/F-12 with 10% horse serum.

To ensure matched cell densities and proportions of neuronal to non-neuronal cell types between conditions within experiments, comparisons were made using sister cultures derived from a single plating.

Experiments were performed using 14-day-old cultures in a test medium of Mg^{2+}-free HBSS. Cultures were exposed to medium containing the drugs tested for 20 min, and then washed twice with medium. Calcium accumulation immediately after and at varying times following drug exposure was assessed in cultures exposed to HBSS containing $^{45}Ca^{2+}$ 20 min prior to, during, and after exposure. Wells were washed three times with saline, cells were lysed with distilled water, and $^{45}Ca^{2+}$ β emmissions in the intracellular contents were counted by scintillation spectroscopy.

Measurement of calcium efflux was accomplished by having $^{45}Ca^{2+}$ present in the medium prior to and during the 20 min exposure period, but at no time thereafter. The amount of $^{45}Ca^{2+}$ remaining in the cells at different time points following the exposure was then determined.

Sequential calcium accumulation was measured by exposing cultures to media containing $^{45}Ca^{2+}$ only during set time intervals following NMDA exposure. Intracellular $^{45}Ca^{2+}$ levels were determined at the end of each interval.

Neuronal injury was assessed qualitatively by light microscopic inspection of the phase contrast appearance of cultures. Quantitative assessments of neuronal injury were made using lactate dehydrogenase (LDH) release as a marker for membrane breakdown and cell death (Koh and Choi, 1987).

Agonist stimulated phosphoinositide formation was determined using cultures incubated for 48 hr in DME/F-12 with 10% horse serum containing 1 μCi ml^{-1} [^{3}H]-myo-inositol. Cultures were washed twice with Mg^{2+}-free HBSS containing 10 mM LiCl (HBSS/LiCl) and incubated at 37°C for 15 min to block inositol phosphate degradation (Allison, Blisner, Holland, Hipps and Shermon, 1976; Berridge, Downes and Hanley, 1982). Agonist exposures of 20 min duration were performed at 37°C in Mg^{2+}-free HBSS/LiCl, and were terminated by replacing the incubation medium with 0.5 ml ice-cold trichloroacetic acid (TCA). Each TCA extract was applied to a Dowex formate anion exchange mini-column. To remove free residual ^{3}H inositol, columns were washed four times with 3 ml 5 mM inositol. ^{3}H inositol phosphates were then eluted with two times 2 ml of 1 M ammonium formate containing 0.1 M formic acid. All fractions were collected, added to 15 ml of scintillation cocktail (Beckman Ready Gel), and counted for tritium on a scintillation spectrophotometer (Packard).

Myo-[2-^{3}H]Inositol: water/ethanol (9:1) solution was obtained from Amersham, Dowex AG1-X8 Anion exchange resin 100–200 mesh formate form was supplied by Biorad, and [(1S,3R)-ACPD] was from Tocris Neuramin Ltd. Glutamic acid, and *N*-methyl-D-aspartic acid (NMDA) were obtained from Sigma. $^{45}Ca^{2+}$ was provided by ICN Biomedicals Inc.

RESULTS

Phosphoinositide formation

Exposure of cerebrocortical cultures to glutamate or (1S,3R)-ACPD for 20 min evoked concentration-dependent increases in [^{3}H]phosphoinositide formation with estimated EC_{50} values of 23 $\pm$ 5 μM and 13 $\pm$ 3 μM, respectively.

NMDA evoked only small responses at concentrations up to 1 mM. Results are summarized in Fig. 1.

Excitatory amino acid-induced calcium accumulation and neuronal death

Exposure of cultures to NMDA or glutamate for 20 min caused concentration dependent increases in $^{45}Ca^{2+}$ accumulation, measured immediately after exposure, and delayed neuronal degeneration. Swelling of neuronal cell bodies and neurites caused by toxic concentrations of these agonists was followed by a progressive cellular disintegration over the following 24 hr. Cellular breakdown was paralleled by a reduction of intracellular $^{45}Ca^{2+}$ content due to

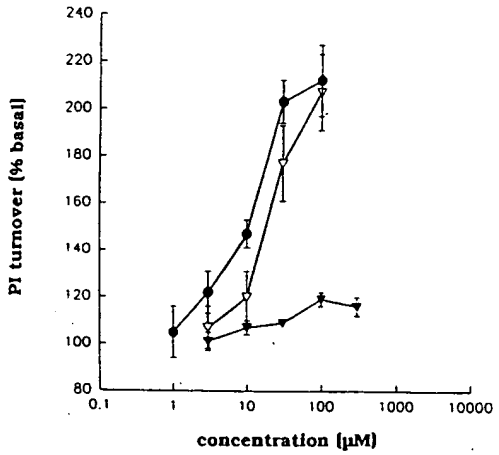

Fig. 1. Concentration-dependent phosphoinositide formation in cerebrocortical cultures in response to a 20 min exposure to (1S,3R)-ACPD (●, $n = 45$), glutamate (▽, $n = 21$) and NMDA (▼, $n = 9$). Values represent means $\pm$ SEM of n determinations for each data point taken from between 3 and 15 separate experiments. Basal phosphoinositide formation was 767 $\pm$ 53 c.p.m.

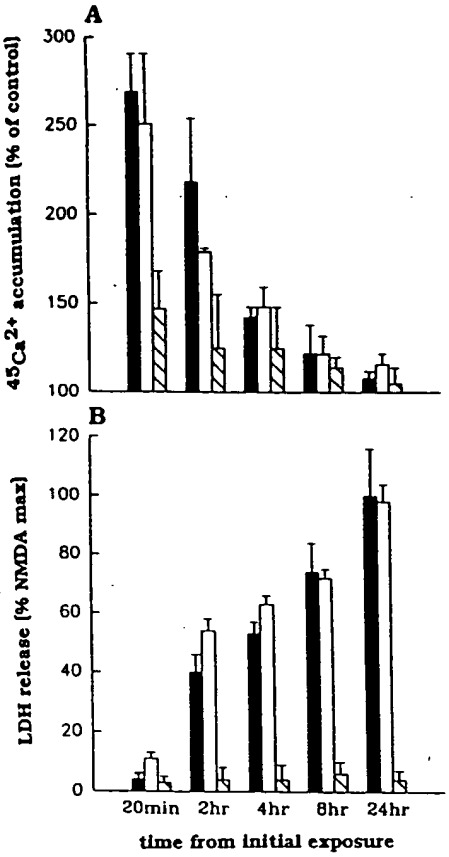

Fig. 2. Time course for: (A) $^{45}Ca^{2+}$ accumulation and (B) LDH release following exposure to 300 μM glutamate (filled bars), 300 μM NMDA (open bars) or 300 μM (1S,3R)-ACPD (hatched bars) for 20 min. Experiments were performed in HBSS containing $^{45}Ca^{2+}$ throughout. All values represent means $\pm$ SEM for $n = 6$. Values for $^{45}Ca^{2+}$ accumulation are expressed as a percentage of sham washed control values (= 100) at each time point. Values for LDH are expressed as a percentage of the mean value in sister cultures exposed to 1 mM NMDA (= 100) at 24 hr after subtraction of mean background values at each time point.

leakage through damaged cell membranes. Estimated LD_{50} values, as measured by LDH release into the media at 24 hr, were 133 $\pm$ 16 μM for glutamate and 122 $\pm$ 13 μM for NMDA.

Cultures exposed to (1S,3R)-ACPD at concentrations up to 1 mM for 20 min, although displaying some increase in $^{45}Ca^{2+}$ accumulation, did not show any signs of cell swelling or delayed neuronal death. A summary of the time course of $^{45}Ca^{2+}$ accumulation and LDH release following exposure of cultures to 300 μM glutamate, 300 μM NMDA or 300 μM (1S,3R)-ACPD (maximally effective concentrations of these agonists for stimulating calcium uptake) is shown in Fig. 2.

Effect of (1S,3R)-ACPD on NMDA or glutamate-induced Ca^{2+} accumulation and cell death

The lethal effects of NMDA were clearly attenuated by the presence of (1S,3R)-ACPD (100 μM) in the media during exposure of cultures to NMDA. (1S,3R)-ACPD protection was most effective against an approximate LD_{50} concentration of NMDA (100 μM), and was characterized by a marked delay in cell degeneration rather than a complete blockade of NMDA neurotoxicity. Results are summarized in Fig. 3.

(1S,3R)-ACPD had no effect on the initial neuronal swelling caused by 100 μM NMDA, however, cells returned to a normal appearance 2–4 hr following exposure rather than remaining swollen and progressively degenerating.

The initial $^{45}Ca^{2+}$ accumulation evoked by 100 μM NMDA was unaffected by (1S,3R)-ACPD. Elevated levels of intracellular $^{45}Ca^{2+}$ persisted until neuronal degeneration occurred between 4 and 8 hr after exposure to NMDA. However, with NMDA and (1S,3R)-ACPD co-exposures, intracellular $^{45}Ca^{2+}$

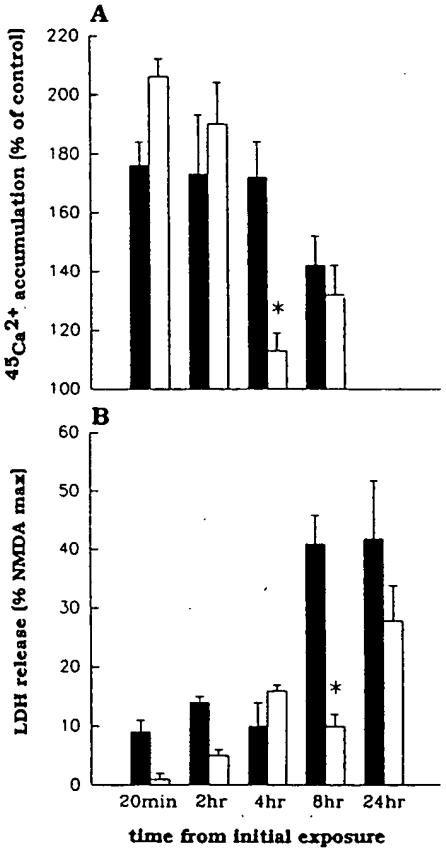

Fig. 3. Time course for: (A) $^{45}Ca^{2+}$ accumulation and (B) LDH release following exposure to 100 μM NMDA alone (filled bars) or in the presence of 100 μM (1S,3R)-ACPD (open bars). All values represent means + SEM for $n = 6$. Values for $^{45}Ca^{2+}$ accumulation are expressed as a percentage of sham washed control values (= 100) at each time point. Values for LDH are expressed as a percentage of the mean value in sister cultures exposed to 1 mM NMDA (= 100) at 24 hr after subtraction of mean background values at each time point. Values significantly less than those for cultures exposed to NMDA alone are shown as * when $P < 0.05$ (Students t-test).

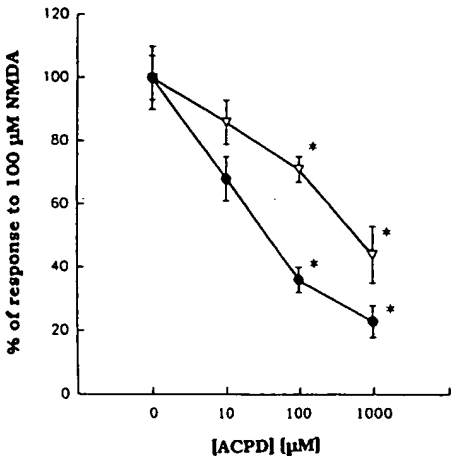

Fig. 4. Concentration-dependent effects of (1S,3R)-ACPD on NMDA-induced delayed $^{45}Ca^{2+}$ accumulation (●) and LDH release (▽). Values represent means ± SEM ($n = 6$) and are expressed as a percentage of $^{45}Ca^{2+}$ accumulation at 4 hr and LDH release at 8 hr following a 20 min exposure to 100 μM NMDA. Values significantly less than those for cultures exposed to NMDA alone are shown as * when $P < 0.05$ (Students t-test).

levels had returned to control values by 4 hr. The decreased $^{45}Ca^{2+}$ levels at 4 hr was not the result of cell death (see Fig. 3).

In a separate series of experiments the neuroprotective effects of (1S,3R)-ACPD were demonstrated to be concentration-dependent, as were its effects on sustained intracellular $^{45}Ca^{2+}$ accumulation. Results are summarized in Fig. 4.

Cultures exposed to higher concentrations of NMDA (300 μM–1 mM) displayed a more rapidly induced cell death (4 hr) which was not attenuated by co-exposure with (1S,3R)-ACPD at concentrations up to 1 mM.

Addition of (1S,3R)-ACPD (100 μM) to the media during exposure to glutamate (10 μM–1 mM) had no effect on the resulting $^{45}Ca^{2+}$ accumulation or delayed cell death (data not shown). The time course of $^{45}Ca^{2+}$ accumulation following exposure to 100 μM glutamate was similar to that seen with 100 μM NMDA in the presence of (1S,3R)-ACPD. Intracellular $^{45}Ca^{2+}$ levels returned to control values at 4 hr post-exposure, this being prior to any increase in LDH release. Immediately following exposure to 100 μM glutamate, cells appeared swollen and remained so until degeneration occurred after a delay of between 8 and 24 hr. This pattern was unaffected by the presence of (1S,3R)-ACPD.

Effects of (1S,3R)-ACPD on calcium efflux and sequential intracellular accumulation following exposure to NMDA

To investigate the mechanism whereby (1S,3R)-ACPD reduced sustained elevations in intracellular $^{45}Ca^{2+}$ evoked by NMDA, the times at which $^{45}Ca^{2+}$ was present in the medium was manipulated to obtain measures of calcium efflux or intracellular Ca^{2+} accumulation at different time points following NMDA exposure. Results are summarized in Fig. 5.

Following $^{45}Ca^{2+}$ accumulation evoked by a 20 min exposure to 100 μM NMDA, intracellular levels of $^{45}Ca^{2+}$ declined over the next 2–8 hr. This decline in intracellular $^{45}Ca^{2+}$ was paralleled by a progressive increase in LDH release into the medium. In the presence of (1S,3R)-ACPD, intracellular levels of $^{45}Ca^{2+}$ followed a similar pattern. However, this reduction in intracellular $^{45}Ca^{2+}$ was not associated with neuronal cell death as assessed by light microscopic examination of cell appearance and by LDH release.

Sequential intracellular $^{45}Ca^{2+}$ accumulation, measured over 2 hr time intervals, remained significantly elevated above control values from 2–6 hr following initial exposure to 100 μM NMDA. The addition of 100 μM ACPD to the exposure medium caused a reduction in this delayed $^{45}Ca^{2+}$ accumulation to levels no greater than those for control cultures.

Effect of pertussis toxin (PTX) pretreatment on (1S,3R)-ACPD neuroprotection

As shown in Fig. 6, pretreatment of cultures for 12 hr with PTX at a concentration of 1 μg ml^{-1} inhibited the protective effect of (1S,3R)-ACPD on NMDA-induced delayed cell death. PTX pretreatment had no effect on the outcome of experiments involving control or NMDA treated cultures.

Role of PKC in (1S,3R)-ACPD attenuation of NMDA activity

The effects of PKC activation on NMDA toxicity and the effects of PKC inhibition on the protective effects of (1S,3R)-ACPD were examined. Results are summarized in Fig. 7.

Addition of PDBu (300 nM) to the medium 10 min prior to and during a 20 min exposure to 100 μM NMDA had no effect on the resulting delayed cell death. The addition of the PKC inhibitor H-7 (200 μM) to the medium 10 min prior to and during a 20 min exposure to 100 μM NMDA in the presence of 100 μM (1S,3R)-ACPD had no effect on (1S,3R)-ACPD neuroprotection against NMDA.

DISCUSSION

Results from the present study provide evidence that in cerebrocortical neurones activation of glutamate metabotropic receptors attenuates NMDA-induced delayed neuronal cell death via a mechanism involving a reduction of sustained intracellular Ca^{2+} accumulation.

Glutamate and (1S,3R)-ACPD evoked concentration-dependent increases in phosphoinositide accumulation with EC_{50} values in the same range as those described in other neuronal preparations

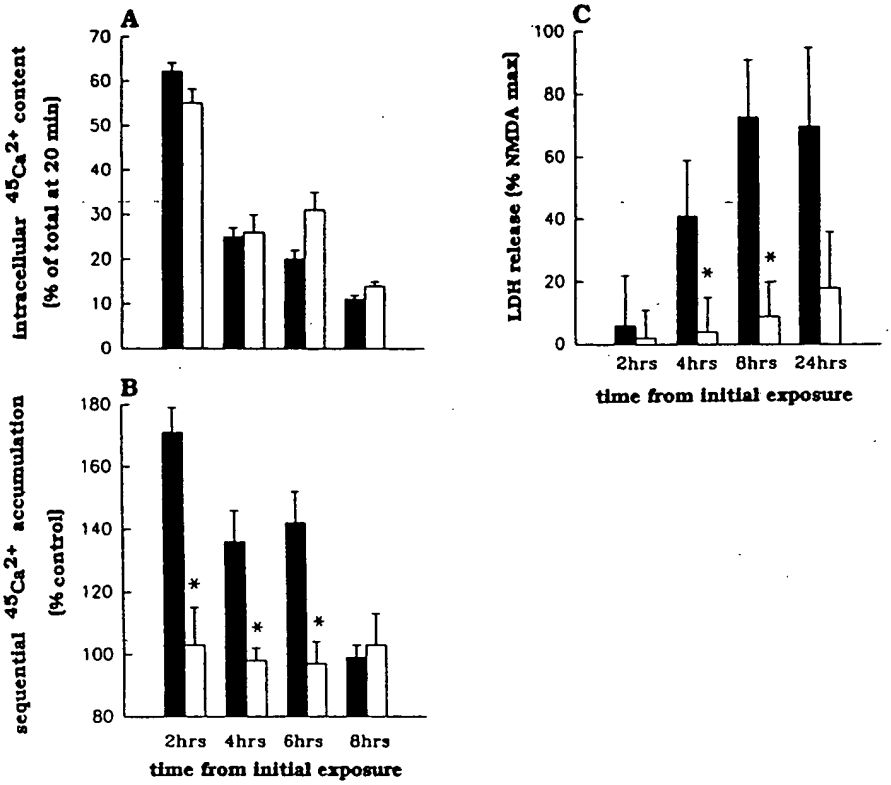

Fig. 5. Time course for: (A) calcium efflux and (B) sequential calcium accumulation following a 20 min exposure to 100 μM NMDA (filled bars) alone or in the presence of 100 μM (1S,3R)-ACPD (open bars). Corresponding values for LDH release are shown in (C). All values represent means + SEM for $n = 6$. In (A) values for $^{45}Ca^{2+}$ accumulation are expressed as a percentage of total $^{45}Ca^{2+}$ influx immediately following exposure to NMDA (= 100). In (B) values for $^{45}Ca^{2+}$ accumulation are expressed as a percentage of $^{45}Ca^{2+}$ accumulation over each time interval in sham washed sister cultures (= 100). Values for LDH are expressed as a percentage of the mean value in sister cultures exposed to 1 mM NMDA (= 100) at 24 hr after subtraction of mean background values at each time point. Values significantly less than those for cultures exposed to NMDA alone are shown as * when $P < 0.05$ (Students t-test).

(Schoepp and Johnson, 1989; Schoepp, Johnson, Smith and McQuaid, 1990b; Masu, Tanabe, Tsuchida, Shigemoto and Nakanishi, 1991). These responses are mediated by distinct metabotropic glutamate receptors and are independent of ionotropic receptor activation (Birrell and Marcoux, 1993). As in other studies (Sladeczek, Pin, Recasens, Bockaert and Weiss, 1985; Nicolletti, Wrolewski and Costa, 1987; Recasens, Guiramand, Nourigat, Sassetti and Devilliers, 1988; Godfrey, Wilkins, Tyler and Watson, 1988; Schoepp and Johnson, 1988; Gonzales and Moerschbaecher, 1989) NMDA produced only weak effects on phosphoinositide accumulation.

A 20 min exposure to NMDA or glutamate evoked concentration-dependent increases in intracellular Ca^{2+} accumulation which persisted after removal of agonist from the culture medium. Sustained elevations in Ca^{2+} were followed by a delayed neuronal cell death at 8 to 24 hr following exposure. The selective metabotropic glutamate receptor agonist (1S,3R)-ACPD, although evoking small increases in intracellular Ca^{2+}, did not cause subsequent neuronal cell death. This is in agreement with the findings of Koh and colleagues (Koh *et al.*, 1991a) who examined the effects of *trans*-ACPD in cortical cultures.

Exposure to NMDA at an approximate LD_{50} concentration caused a delayed cell death that was attenuated in a concentration-dependent manner by co-administration of (1S,3R)-ACPD. This neuroprotective effect was associated with a reduction in the sustained Ca^{2+} accumulation caused by NMDA. Independent measurement of Ca^{2+} efflux and intracellular accumulation suggested that this reduced Ca^{2+} accumulation resulted from both the efflux of Ca^{2+}, without associated cell death, and a blockade of delayed (post-exposure) intracellular Ca^{2+} accumulation.

Ca^{2+} accumulation and cell death following exposure to an approximate LD_{50} concentration of glutamate had a similar time course to that seen following exposure to NMDA with (1S,3R)-ACPD. Intracellular Ca^{2+} accumulation declined before the onset of delayed cell damage and neuronal death.

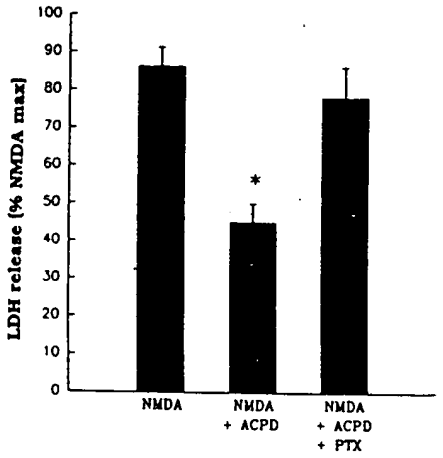

Fig. 6. Effect of PTX pretreatment on the neuroprotective effects of (1S,3R)-ACPD against NMDA toxicity. Bars represent mean + SEM ($n = 6$) for LDH release measured at 8 hr from initial exposure to either 100 μM NMDA, 100 μM NMDA in the presence 100 μM (1S,3R)-ACPD or 100 μM NMDA in the presence of 100 μM (1S,3R)-ACPD following a 12 hr pretreatment with PTX. Values are expressed as a percentage of the mean value in sister cultures exposed to 1 mM NMDA (= 100) at 24 hr after subtraction of mean background values. Values significantly less than those for cultures exposed to NMDA alone are shown as * when $P < 0.05$ (Students t-test).

Exposure of cultures to (1S,3R)-ACPD together with glutamate had no efect on subsequent Ca^{2+} accumulation or cell death. This result was not surprising since, at the concentration used, glutamate causes maximal stimulation of phosphoinositide turnover in these cultures. Thus, the presence of (1S,3R)-ACPD together with glutamate will not cause any further activation of metabotropic glutamate receptors and would not be expected to attenuate glutamate toxicity.

In cultures exposed to NMDA, Ca^{2+} continued to accumulate intracellularly after removal of NMDA from the culture medium. Evidence exists for the involvement both of NMDA (Choi, Koh and Peters, 1988) and non-NMDA (Koh, Goldberg, Hartley and Choi, 1990; Garthwaite and Garthwaite, 1991a, b) inotropic receptors in the delayed phase of glutamate toxicity. Delayed Ca^{2+} influx may occur following extracellular accumulation of endogenous glutamate released by depolarized or damaged neurones, and as a result of the failure or reversal of glutamate uptake systems in neurones or astrocytes (Nicholls and Attwell, 1990). In cultures exposed to NMDA in the presence of (1S,3R)-ACPD, continued Ca^{2+} accumulation did not occur, suggesting that activation of metabotropic glutamate receptors interferes with these processes and may prevent sustained glutamate release, or block continued intracellular accumulation of Ca^{2+} following activation of ionotropic glutamate receptors.

In studies using Ca^{2+} sensitive fluorescent dyes, long-lasting elevations in free intracellular Ca^{2+} are considered to be required for subsequent neuronal cell death (Ogura, Miyamoto and Kudo, 1988; Wahl, Schousboe, Honore and Drejer, 1989; Manev, Favaron, Guidotti and Costa, 1989; Glaum, Scholz and Miller, 1990; Ciardo and Meldolesi, 1991). Recently, it was suggested that following short periods of glutamate exposure, return of elevated free intracellular Ca^{2+} to basal levels may be indicative of prolonged neuronal survival (Randall and Thayer, 1992; Dubinsky, 1993). The ability of cells to survive a large Ca^{2+} evoked by glutamate may result from the activation of Ca^{2+} buffering mechanisms, such as Ca^{2+} extrusion systems, which may occur as a result of metabotropic glutamate receptor stimulation.

In the present study pretreatment with PTX inhibited the protective effects of (1S,3R)-ACPD. Metabotropic glutamate receptor-stimulated phosphoinositide turnover in these cultures is inhibited by PTX pretreatment (Birrell and Marcoux, 1993), suggesting that activation of phospholipase C may be involved in mediating the protective effects of (1S,3R)-ACPD.

Stimulation of neuronal membrane phospholipid metabolism results in the release of Ca^{2+} from intracellular stores and activation of PKC (Berridge, 1987). Increases in intracellular free Ca^{2+} evoked via release from intracellular stores are not as high as those produced by NMDA receptor activation (Deboni and Crapper-McLachlan, 1985; McMillan, Pritchard and Miller, 1990). However, release of calcium from certain intracellular pools

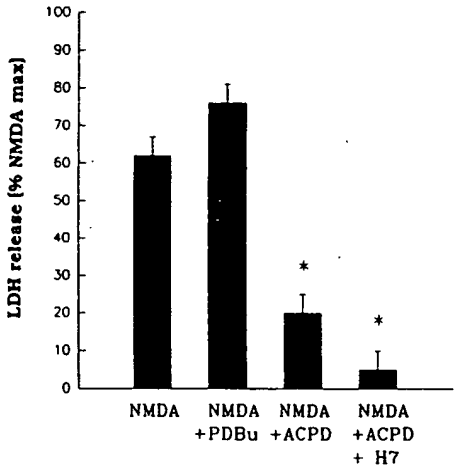

Fig. 7. Effects of PDBu (300 nM) on NMDA (100 μM) toxicity and H-7 (200 μM) on the neuroprotective effect of (1S,3R)-ACPD (100 μM). All values represent means + SEM ($n = 6$) for LDH release measured at 8 hr from initial exposure to either NMDA, NMDA in the presence of PDBu, NMDA in the presence of (1S,3R)-ACPD or NMDA in the presence of (1S,3R)-ACPD and H-7. Values are expressed as a percentage of the mean value in sister cultures exposed to 1 mM NMDA (= 100) at 24 hr after subtraction of mean background values at each time point. Values significantly less than those for cultures exposed to NMDA alone are shown as * when $P < 0.05$ (Students t-test).

is important for localized increases in Ca^{2+} [see Miller (1992)] which may selectively influence cellular processes related to Ca^{2+} homeostasis and/or calcium-dependent phosphorylation of membrane proteins.

Protective effects of metabotropic receptor activation against NMDA toxicity described previously by Koh *et al.* (1991b) were partially blocked by the PKC inhibitor H-7. In the present study H-7, applied at the same concentration as Koh *et al.*, had no inhibitory effect on (1S,3R)-ACPD neuroprotection. In addition, incubation with PDBu, at concentrations known to cause maximal activation of PKC in other neuronal preparations (see Shearman, Sekiguchi and Nishizuka, 1980), had no effect on NMDA toxicity. H-7 is a relatively specific inhibitor of PKC (Hidaka, Inagaki, Kawamoto and Sasaki, 1984), and differences in the role played by PKC between the two studies may result from variations in the culture systems used. It should also be noted that in some culture systems high levels of PKC activation causes neuronal degeneration (Mattson, 1991).

In addition to those metabotropic glutamate receptor subtypes linked to activation of phospholipase C or to inhibition of cAMP accumulation [see Nakanishi (1992)], in other preparations activation of metabotropic glutamate receptors is associated with arachidonic acid release (Aramori and Nakanishi, 1992), stimulation of phospholipase D (Boss and Conn, 1992) or increased cAMP accumulation (Winder and Conn, 1992). Transduction through more than one of these pathways is mediated through PTX sensitive G-proteins, and it is possible that the effects of PTX seen in this study may be occurring via any number of these pathways. In addition, indirect effects of PTX on other G-proteins (Gilman, 1987) cannot be excluded.

Although results from the present study are in agreement with those of other workers showing a protective effect of metabotropic receptor activation on NMDA neurotoxicity (Koh *et al.*, 1991b; Siliprandi *et al.*, 1992), Schoepp and coworkers (Sacaan and Schoepp, 1992) have demonstrated a neurotoxic effect of (1S,3R)-ACPD *in vivo* following intrahippocampal injection. However, in this preparation the neurotoxic effects of (1S,3R)-ACPD are blocked by NMDA receptor antagonists suggesting that activation of metabotropic receptors is not in itself directly responsible for the observed cell death. Such apparently contradictory results may be explained by the existence of a family of metabotropic receptors, each subserving a separate function and being expressed in a range of different cell types [see Schoepp and Conn (1993)].

In conclusion, activation of metabotropic glutamate receptors attenuates NMDA neurotoxicity in primary cerebrocortical cultures via a reduction in delayed intracellular Ca^{2+} accumulation. Inhibition of sustained Ca^{2+} accumulation following exposure to NMDA results both from calcium efflux, not associated with cell death, and from a blockade of delayed intracellular Ca^{2+} accumulation. Identification of the intracellular mechanisms responsible for the effects described here await the further development of more selective agonists and antagonists for the metabotropic glutamate receptor subtypes.

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Metabotropic Glutamate Receptors Negatively Coupled to Adenylate Cyclase Inhibit *N*-Methyl-D-aspartate Receptor Activity and Prevent Neurotoxicity in Mesencephalic Neurons *In Vitro*

Exhibit T (10/644, 645)

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SUMMARY

The functional effects of G protein-linked glutamate receptor activation have been studied in mouse mesencephalic neurons *in vitro*. We have been able to identify two receptor classes, one linked to phosphoinositide hydrolysis and another that inhibits adenylate cyclase. The agonist (1*S*,3*R*)-aminocyclopentane-1,3-dicarboxylate (ACPD) affected the two responses with similar potency (EC_{50} = 2 and 7 μM, respectively). In contrast, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine selectively decreased adenylate cyclase activity (EC_{50} = 150 nM), without interfering with the phosphoinositide pathway. Activation of ion channel-linked glutamate receptors in mesencephalic neurons leads to cGMP formation. In this study, we demonstrate that cell pretreatment with ACPD or (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine prevented, in a dose-dependent fashion, *N*-methyl-D-aspartate (NMDA)-induced cGMP formation but not the kainate-stimulated response. The pharmacological profile suggests that receptors that are negatively coupled to adenylate cyclase are responsible for this effect. Coexposure of neurons to ACPD and Ba^{2+}, a K^+ channel blocker, counteracted the ACPD-induced blockade of NMDA receptors, suggesting that activation of K^+ conductances could be involved in the post-transduction events triggered by metabotropic receptors in the mesencephalon. Neuronal treatment with NMDA for 10 min caused a reduction in mitochondrial activity. Direct inhibition of nitric oxide synthase with the inhibitor N^G-nitro-L-arginine or removal of extracellular nitric oxide with reduced hemoglobin did not prevent this metabolic impairment, thus excluding a role for nitric oxide in this test for excitotoxicity. On the contrary, the mitochondrial function was maintained when neurons exposed to NMDA were preincubated with metabotropic receptor agonists. To summarize, our results suggest that metabotropic receptors that are negatively coupled to adenylate cyclase exert modulatory control specifically on NMDA receptor activity. This event could also contribute to the reduction of neurotoxic effects due to NMDA receptor hyperactivity.

The mesencephalic nuclei substantia nigra and ventral tegmental area receive excitatory glutamatergic afferent innervation mainly from the frontal cortex (1, 2) and from the subthalamic (3, 4) and peduncolopontine (5) nuclei. Functional investigations of the role of glutamate in these areas have been carried out both in mesencephalic slices and in primary neuronal cultures (6–11). These studies focused primarily on the effects of ion channel-linked (ionotropic) GluRs of the NMDA and AMPA/kainate subtypes (12) on dopaminergic neurons. In addition, depolarization of substantia nigra dopaminergic cells was observed after stimulation with ACPD, which elicited a slow inward Na^+ current (13). This ligand is selective for other GluRs, which are referred to as mGluRs because of their link to G proteins and intracellular transducers. The recent cloning of mGluR genes revealed a wide heterogeneity of receptor proteins (14), and it is now known that these receptors influence neuronal activity through modulation of a variety of intracellular events (extensively reviewed in Refs. 15 and 16).

Interest concerning the function of mGluRs in the basal ganglia is now growing, because mRNAs for different mGluR types have been detected in distinct neuronal populations, by *in situ* hybridization analysis (17, 18). The substantia nigra pars compacta expresses moderate levels of mGluR1 (at the dopaminergic cell level) and low levels of mGluR4. mGluR3 is present only in neurons and glial cells of the pars reticulata.

ABBREVIATIONS: GluR, glutamate receptor; ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate; CCGI, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)-glycine; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; PI, phosphoinositide(s); InsP, inositol phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; Quis, quisqualate; AP3, 2-amino-3-phosphonopropionate; NO, nitric oxide; TCA, trichloroacetic acid; RIA, radioimmunoassay.

Both substantia nigra regions were labeled by the probe recognizing mGluR5, whereas no staining was detectable for mGluR2 message (17). RNA for mGluR7 was also detected (18).

Endogenous glutamate, released in the ventral mesencephalon, could simultaneously activate ionotropic GluRs and mGluRs, and the neuronal activity may be the outcome of their mutual modulation through the generation of intracellular signals. We focused our attention on the possible interactions between ion channel- and G protein-linked GluRs in mouse mesencephalic primary cultures enriched in substantia nigra and ventral tegmental area neurons. By measuring second messenger levels, we provide evidence for multiple intracellular pathways triggered by the diverse GluR classes. Notably, we demonstrate that stimulation of mGluRs that inhibit adenylate cyclase causes a negative modulation of NMDA receptor activity.

It has been suggested that persistent and excessive stimulation of NMDA receptors could contribute to the dopaminergic cell damage that occurs with age and in Parkinson's disease (19, 20). A dysfunction in glutamate release could also be responsible for the alteration of the neuronal circuits connecting the ventral tegmental area to the prefrontal cortex that is observed in psychotic patients (20). In this work, we used mesencephalic cell cultures as a model to investigate whether glutamatergic agonists may cause neuronal damage in this brain region. Our results show that NMDA receptor hyperactivity brought about a precocious mitochondrial impairment in mesencephalic neurons and that the neurotoxic action of NMDA could be prevented by mGluR activation.

Experimental Procedures

Materials. Tissue culture media were from Flow Laboratories (Milan, Italy). ^{125}I-cGMP and ^{125}I-cAMP RIA kits and *myo*-[2-^{3}H]-inositol were from Amersham International (Buckinghamshire, UK). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. Excitatory amino acid ligands were from Tocris (Bristol, UK).

Mesencephalic neuronal cultures. Neurons were obtained from Swiss albino mouse fetuses (embryonic day 13; Charles River, Calco, Italy) by dissection of the anteroventral part of the mesencephalon, according to the method of Prochiantz *et al.* (21). The dissociated cells were plated in 33-mm Falcon culture dishes that had been coated first with 1.5 μg/ml polyornithine and then with 7% (v/v) fetal calf serum in Hanks' salts. The culture medium was composed of a mixture (1:1) of Dulbecco's minimal essential medium and F-12 medium, supplemented with glucose (33 mM), glutamine (2 mM), and HEPES (5 mM). Fetal calf serum was replaced by a mixture of hormones, protein, and salts composed of insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenium salt (30 nM). Under these conditions non-neuronal cell proliferation is greatly reduced and glial cells represent <5% of the total population (22). Immunocytochemistry revealed the presence of tyrosine hydroxylase- and glutamate decarboxylase-positive cells (data not shown).

Determination of cGMP. After 7–9 days *in vitro*, the growth medium was removed and cells were washed twice with a modified Krebs-Ringer medium buffered with HEPES-NaOH, pH 7.4, which contained 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 12 mM glucose. Incubation medium also included 1 mM IBMX, a blocker of cGMP and cAMP phosphodiesterases, and 1 μM tetrodotoxin, to block plasma membrane depolarization and endogenous neurotransmitter release. Experiments were performed at room temperature. After a 10-min preincubation to allow IBMX incorporation and a 5-min treatment with mGluR agonists, neurons were exposed to NMDA and kainate for 1 min, at which time the production of cGMP was maximal (23). NMDA studies were performed in Mg^{2+}-free buffer, preceded by a brief wash of neurons with the same buffer. The reaction was terminated by addition of cold perchloric acid (0.4 N). After at least 15 min in the cold, the preparations were carefully collected, transferred to plastic Eppendorf tubes, and centrifuged at 8000 × *g* for 5 min. The supernatant was transferred to other tubes and neutralized with K_2CO_3, and aliquots were tested for cGMP content by using a commercially available ^{125}I-cGMP RIA kit, as reported previously (23). The pellets from the original centrifugation were dissolved in 0.5 N NaOH and protein content was measured by the method of Lowry *et al.* (24), with bovine serum albumin as the standard.

Determination of cAMP. After preincubation with IBMX for 10 min, neurons were exposed to mGluR agonists for 10 min at room temperature. Experiments were performed either in the presence or in the absence of 10 μM forskolin. Samples were treated as described for cGMP determination. Aliquots of the supernatants were tested for cAMP content by using a commercially available ^{125}I-cAMP RIA kit.

PI hydrolysis. After 7–8 days *in vitro*, neurons were incubated for 24 hr with 2 μCi/ml *myo*-[2-^{3}H]inositol (specific activity, 17 Ci/mmol; Amersham) to label plasma membrane PI. After two washes with the incubation buffer, neurons were stimulated for 10 min with the agonists, in the presence of 5 mM LiCl (to avoid inositol monophosphate degradation) and 1 μM tetrodotoxin. The reaction, conducted at room temperature, was terminated by treatment of the monolayers with 15% ice-cold TCA. After at least 15 min in the cold, the preparations were carefully collected, transferred to plastic Eppendorf tubes, centrifuged at 8000 × *g* for 5 min, and processed as reported previously (22), to separate the pool of labeled InsPs obtained by PI hydrolysis. Briefly, after TCA extraction with diethyl ether, samples were neutralized with sodium tetraborate and the [^{3}H]InsPs were bound to AG1-X8 resin (Bio-Rad) and together eluted with 0.1 M formic acid/1.2 M ammonium formate. The pellets from the initial TCA precipitation were dissolved in 0.5 N NaOH and processed for measurement of the protein content.

NMDA toxicity. Mesencephalic neurons were used for neurotoxicity studies after 7–8 days *in vitro*. After replacement of the growth medium with Mg^{2+}-free buffer, NMDA (plus 1 μM glycine and 1 μM tetrodotoxin) was added to the neuronal cultures for 10 min at room temperature. The NMDA-containing solution was then removed and the original medium was added back to the dishes, which were returned to a 37° incubator, containing 5% carbon dioxide, for various times (30 min, 1 hr, 3 hr, or 24 hr) to allow manifestation of injury. Incubation for 1 hr at 37°, after the 10-min NMDA exposure, was the shortest period in which toxicity was manifested. This incubation time was used in all experiments reported herein. Rinsing of the cells before replacement of their medium or addition of fresh growth medium was found to greatly damage the cultures. Therefore, such manipulations were not routinely performed and the conditioned medium of each dish was set aside during the exposure to NMDA and added back to the dishes. To quantify early cytological damage, mitochondrial activity was assessed by a colorimetric assay with MTT (25).

MTT assay. Yellow MTT is converted to the blue formazan product only by metabolically active mitochondria, and the absorbance is directly proportional to the number of viable cells (25). MTT was added to the cultures at 0.5 mg/ml (final concentration) 1 hr after NMDA treatment, and color was allowed to develop for an additional 1 hr. An equal volume of 0.08 N HCl/isopropanol was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into spectrophotometric cuvettes and read at a test wavelength of 570 nm and a reference wavelength of 630 nm. Data are expressed in arbitrary units, where the absorbance difference (absorbance at 570 nm minus absorbance at 630 nm) in the control samples equals 1 unit.

Statistical analysis. Results are expressed as means ± standard errors. The statistical significance of the differences between means was analyzed by the Student *t* test.

Results

mGluR activation inhibits NMDA receptor-induced cGMP formation in mesencephalic neurons. NMDA-induced cGMP formation was completely blocked when mesencephalic neuronal cultures were pretreated for 5 min with the selective mGluR agonists ACPD (100 μM), CCGI (1 μM) (26), or DHPG (100 μM) (27) before stimulation for 1 min with NMDA (Fig. 1). In contrast, ACPD and CCGI did not affect cGMP formation stimulated by kainate (Fig. 1). Dose-response curves obtained with ACPD and CCGI revealed that these two compounds may affect NMDA receptor activity at very low doses (Fig. 2), with half-maximal effects at approximately 0.5 μM and 10 nM, respectively. The 5-min pretreatment with CCGI had a negligible effect on basal cGMP levels, whereas, in some experiments, ACPD slightly decreased basal values as well (about 10%). Preincubation for 5 min with Quis (10 μM), tested in a single experiment, potently increased cGMP levels, both under control conditions and in the presence of 100 μM NMDA (115% and 169% increases above basal levels, respectively). Quis may also affect AMPA/kainate receptors (12) and, indeed, the activation of this ionotropic GluR class may account for the results obtained. For this reason, the effects of this nonselective agonist on cGMP levels were not further investigated in this study.

To understand which mechanism coupled mGluR with NMDA receptor activity, we exposed neurons to the highest concentration of ACPD tested (100 μM) together with Ba^{2+} at 100 μM, a concentration at which it blocks some K^+ conductances (28–30). Ba^{2+}, by itself, did not modify basal or

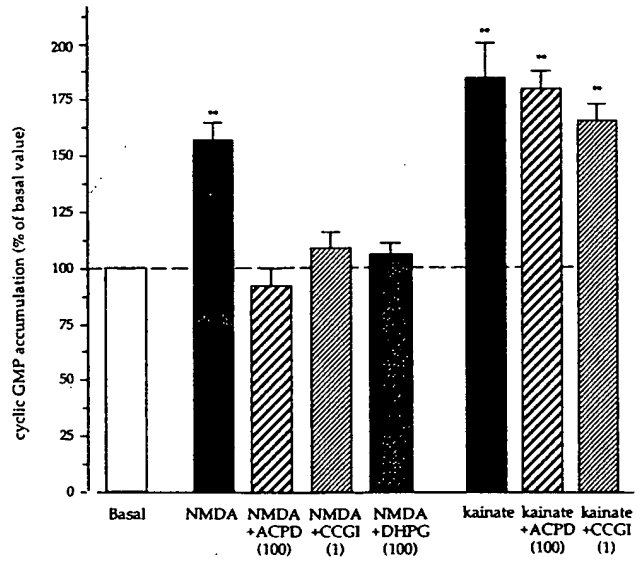

Fig. 1. Effects of mGluR agonists on NMDA- and kainate-induced cGMP accumulation in mesencephalic neurons. After 5-min pretreatment with the mGluR agonists (at the concentrations given in parentheses, in μM), cells were incubated for 1 min with or without NMDA or kainate (both at 100 μM). Results are shown as percentages of basal cGMP values (2.09 ± 0.24 pmol/mg of protein) and are the means ± standard errors of three to five experiments performed in triplicate with different cell preparations. **, $p < 0.01$ in a one-tailed *t* test, compared with controls.

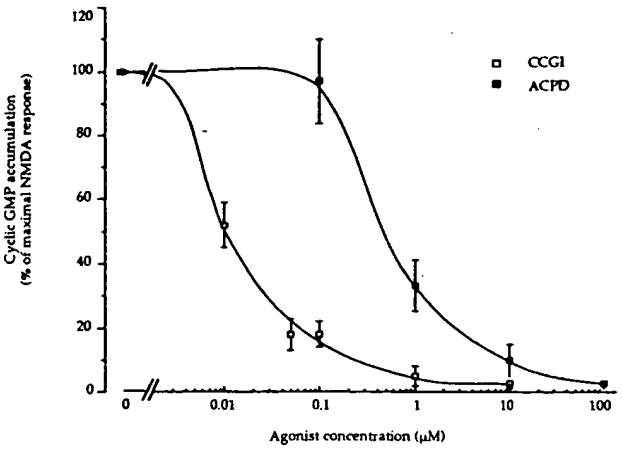

Fig. 2. Dose-response curves for mGluR agonist effects on NMDA-induced cGMP accumulation in mesencephalic neurons. After 5-min pretreatment with the mGluR agonists (at the concentrations indicated), cells were incubated for 1 min with or without NMDA (100 μM). Results are shown as percentages of the maximal NMDA response, which was 60 ± 10% above basal cGMP values (3.20 ± 0.31 pmol/mg of protein), and are the means ± standard errors of at least three experiments performed in triplicate with different cell preparations. EC_{50} values for inhibition were 10 nM for CCGI and 500 nM for ACPD.

NMDA-induced cGMP accumulation, but it completely abolished the negative modulation exerted by ACPD on NMDA receptor activity (Fig. 3). General blockers such as tetraethylammonium and 4-aminopyridine (used in the 0.1–10 mM range) or iberiotoxin (1–10 nM), which is selective for the big-conductance, Ca^{2+}-dependent, K^+ channels (31), gave controversial results when added together with mGluR ligands and also greatly affected basal cGMP values (data not shown).

mGluRs modulate second messenger levels in mesencephalic neuronal cultures. To identify which mGluR subtype was responsible for the inhibition of the NMDA-mediated cGMP formation, we investigated the ability of

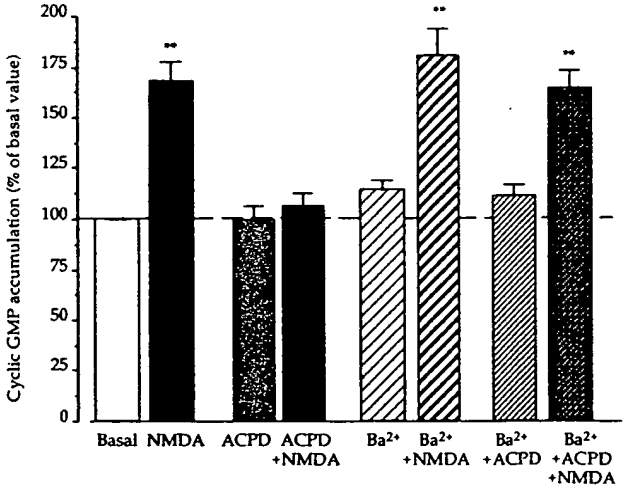

Fig. 3. Ba^{2+} prevention of the ACPD-induced inhibition of NMDA receptor activity. After 5-min pre-exposure to ACPD and/or Ba^{2+} (both at 100 μM), cells were treated for 1 min with or without NMDA (100 μM). Results are shown as percentages of basal cGMP values (2.62 ± 0.28 pmol/mg of protein) and are the means ± standard errors of four experiments performed in triplicate with different cell preparations. **, $p < 0.01$ in a one-tailed *t* test, compared with basal values.

selective mGluR ligands to modulate the intracellular levels of the two second messengers, i.e., InsPs and cAMP (15). As can be seen in Fig. 4, an increase in [^{3}H]InsPs could be induced by ACPD, Quis, and DHPG, with similar potencies. The mGluR agonist CCGI did not affect PI hydrolysis in the concentration range used (Fig. 4). The noncompetitive antagonist AP3 (15) at 10 μM reduced responses to ACPD or Quis by 45–50% (Table 1).

The mGluR agonists were tested for their effects on adenylate cyclase. To compare the influence of mGluRs on this second messenger pathway with their effects on NMDA receptors, we evaluated whether basal cAMP values were modified by the diverse agonists. ACPD and CCGI decreased basal cAMP levels, with E_{max} values for inhibition of 23% at 500 μM ACPD and 36% at 5 μM CCGI (Fig. 5A). DHPG at 100 μM, the concentration used in the studies of NMDA receptor activity shown in Fig. 1, reduced basal cAMP levels by 30 ± 2% (data not shown), whereas the effect of Quis was always stimulatory (Fig. 5A). In Fig. 5B, we report the dose-dependent reduction in cAMP levels observed with mGluR ligands when the enzyme was activated by forskolin (10 μM). The rank order of potency was CCGI ≫ ACPD > DHPG = Quis. The inhibition of forskolin-induced responses was never complete and reached 43% with 5 μM CCGI or 48% with 500 μM ACPD. The maximal inhibition with Quis or DHPG was about 35%. At a higher concentration (500 μM) Quis had a stimulatory effect (data not shown). As mentioned above, however, the latter compound may also affect ionotropic GluRs and, therefore, the results obtained must be carefully interpreted, because more complex intracellular mechanisms may be involved.

mGluR activation protects mesencephalic neurons from NMDA-induced toxicity. L-Glutamate and, more selectively, NMDA and kainate could damage mesencephalic neurons when administered at high concentrations. In particular, in a subpopulation of neurons kainate (100–500 μM) produced acute toxic phenomena, characterized by neuronal swelling that was evident after 5 min of exposure to the neurotoxin (data not shown). NMDA did not cause appreciable morphological changes in 1–3 hr, as determined by visual observation of the cultures. However, its toxicity was detectable by measurement of the ability of neuronal mitochondria to convert the MTT salt into blue formazan (25). Metabolic impairment was already evident in the mesencephalic neurons at 1 hr after their treatment with NMDA for 10 min. This NMDA-related toxicity was dose dependent (Fig. 6A), with maximal effects being seen at 500 μM, and it was completely antagonized by the NMDA channel blocker MK-801 (10 μM) (12), added for 10 min together with NMDA (Fig. 6B).

Treatment with ACPD has been shown to exert neuroprotection from NMDA toxicity in various neuronal cell types (32, 33). We investigated whether the mGluR-mediated modulation of NMDA receptor activity could play a neuroprotective role also in our neuronal system. Indeed, ACPD, CCGI, and DHPG at concentrations that inhibited NMDA-induced cGMP formation (Fig. 1) completely prevented NMDA injury of mesencephalic neurons (Fig. 7).

It has been proposed that excessive release of NO may be neurotoxic in cultured neurons (34). Because the NO-cGMP pathway triggered by NMDA receptors was blocked by mGluR agonists (Fig. 1), we tested whether their mechanism of neuroprotection was dependent on NO synthase inhibition. Preincubation of neurons with the NO synthase blocker N^G-nitro-L-arginine, at doses that completely inhibited cGMP formation (23), did not protect neurons from the toxic effects of NMDA. In addition, 100 μM N^G-nitro-L-arginine was toxic by itself (Fig. 8). Reduced hemoglobin (10 μM), which acts as a chelator of NO released in the extracellular space (33), was also unable to block NMDA-dependent mitochondrial damage and was toxic by itself (Fig. 8).

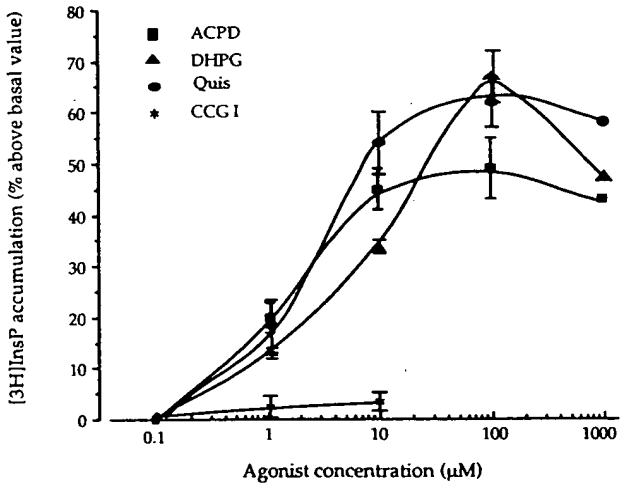

Fig. 4. Dose-response curves for mGluR agonist effects on [^{3}H]InsP formation in mesencephalic neurons. Cells labeled with *myo*-[2-^{3}H]-inositol were stimulated for 10 min with ACPD, CCGI, DHPG, or Quis at the indicated doses. Values, expressed as percentages of basal values (1925 ± 177 cpm/mg of protein), are the means ± standard errors of at least three independent experiments performed in triplicate with different neuronal preparations. Agonists were tested at 1 mM only once. CCGI was ineffective at all concentrations tested; EC_{50} values for the other agonists were 2 μM for ACPD, 2.5 μM for Quis, and 10 μM for DHPG.

TABLE 1

Effects of mGluR ligands on [^{3}H]InsP formation in mesencephalic neurons *in vitro*

Neurons were preincubated for 5 min in the presence or absence of AP3 and were then treated for 10 min with the mGluR agonists ACPD or Quis. Values are expressed as percentages of basal values (2588 ± 280 cpm/mg of protein) and are the averages ± standard errors of at least three independent experiments performed in triplicate.

	[^{3}H]InsP accumulation
	% of basal
Basal	100
ACPD (10 μM)	148 ± 5[a]
Quis (10 μM)	144 ± 4[a]
AP3 (10 μM)	107 ± 8
AP3 + ACPD (both 10 μM)	126 ± 2[b]
AP3 + Quis (both 10 μM)	122 ± 4[b]

[a] $p < 0.01$ in a one-tailed t test, compared with basal levels.
[b] $p < 0.05$.

Discussion

In this study, we provide new evidence for a functional role of mGluRs in mesencephalic neurons *in vitro*. Our main finding is that the stimulation of mGluRs may negatively control the activity of another GluR type, namely the NMDA subtype.

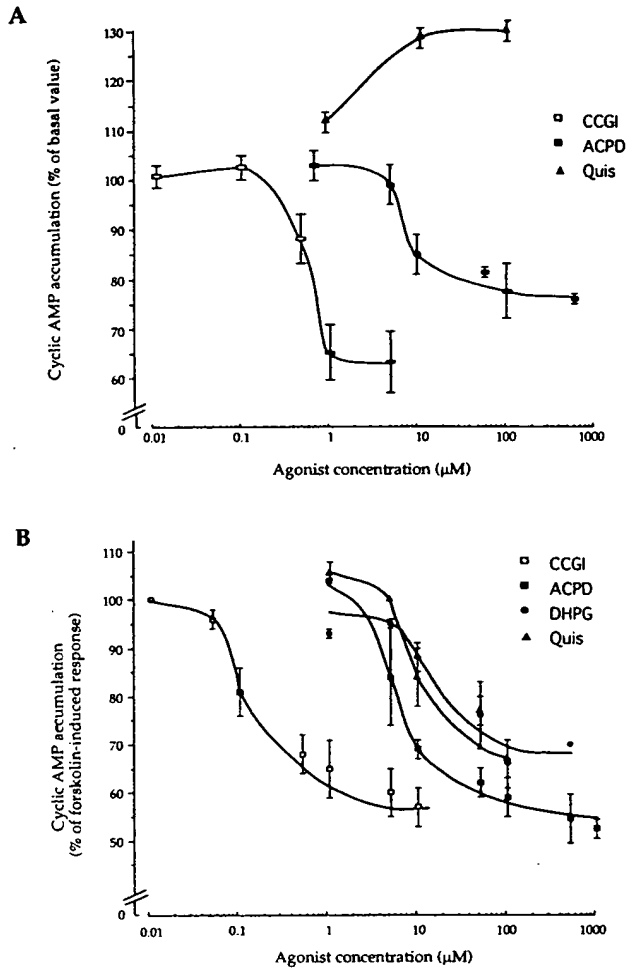

Fig. 5. Concentration-dependent inhibition by mGluR agonists of adenylate cyclase activity in mesencephalic neurons. Neurons were incubated for 10 min under basal conditions or in the presence of forskolin (10 μM), with increasing concentrations of mGluR ligands. A, Effects of ACPD, CCGI, or Quis on basal cAMP levels. Values are expressed as percentages of basal cAMP values (44.48 ± 4 pmol/mg of protein, means ± standard errors of three experiments). B, Effects of ACPD, CCGI, DHPG, or Quis on forskolin-induced cAMP accumulation. Values are expressed as percentages of forskolin-stimulated cAMP levels (494 ± 70 pmol/mg of protein) and are the means ± standard errors of three or four independent experiments performed in triplicate with different neuronal preparations. Under basal conditions (A) Quis was stimulatory, whereas the EC_{50} values of inhibition for the other agonists were 7 μM for ACPD and 500 nM for CCGI. In the presence of forskolin (B) the EC_{50} values were 7 μM for ACPD, 150 nM for CCGI, 12 μM for Quis, and 20 μM for DHPG.

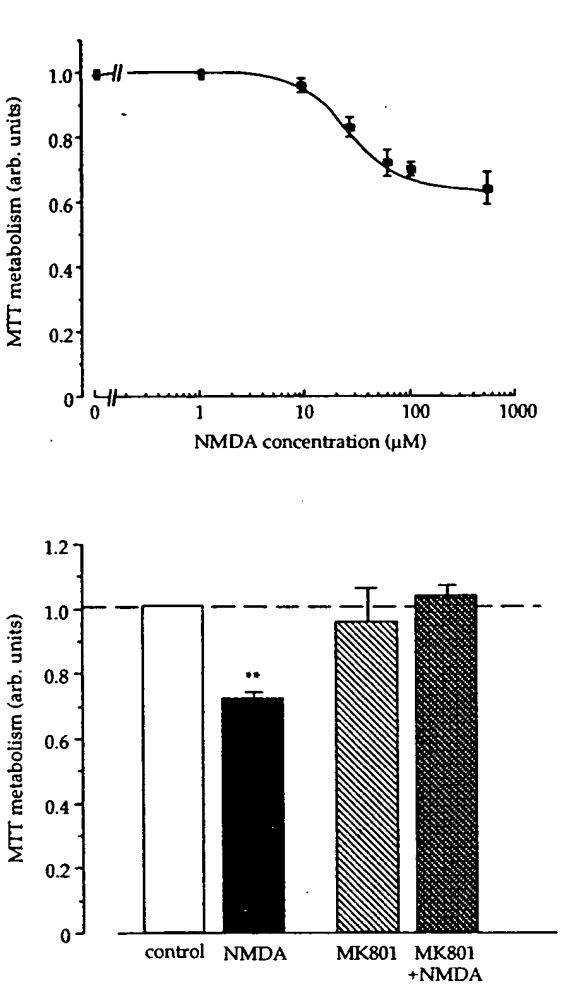

Fig. 6. NMDA-induced toxicity in mesencephalic neuronal cultures. A, Dose-response curves for NMDA effects on MTT conversion, after 10-min incubation under control conditions or in the presence of increasing concentrations of NMDA (1–500 μM). B, Effect of the NMDA antagonist MK-801 (10 μM), added to the cultures during 10-min exposure to 100 μM NMDA. After replacement of NMDA-containing buffer with the original growth medium, dishes were returned to the incubator for 1 hr and then MTT was added for an additional 1 hr, as described in detail in Experimental Procedures. Data (absorbance difference, i.e., absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of at least three independent experiments performed in quadruplicate. **, $p < 0.01$ for 100 μM NMDA in a one-tailed t test, compared with controls.

We have previously shown that, in mesencephalic neurons, ionotropic GluR activation stimulates intracellular generation of cGMP (23). In the present work, we used this biochemical variable as a tool to estimate NMDA or kainate receptor activity after neuronal exposure to selective mGluR agonists. Whereas kainate receptor-induced cGMP formation remained unaffected by ACPD or CCGI, these compounds caused a dose-dependent block of the NMDA response (Figs. 1 and 2).

The recent discovery of selective agonists for the various mGluRs added new information regarding their function in normal and pathological neuronal transmission (reviewed in Ref. 15). In general, slow excitatory events and neurotoxic action appear to be related to PI-linked mGluR activation (35, 36), whereas the inhibitory effects of mGluRs have been considered secondary to the activity of receptors negatively linked to adenylate cyclase (15). An exception is represented by cerebellar neurons, where PI-linked mGluRs reduce cellular excitability (37) and NMDA-related toxicity (33). Our goal was to identify the subtypes functionally expressed in mesencephalic neuronal preparations and the concentration ranges for selective mGluR agonists at which selectivity for a particular subtype was manifested.

Our results clearly demonstrate that mesencephalic neurons *in vitro* express both mGluRs that stimulate PI hydrolysis and mGluRs that inhibit adenylate cyclase (Figs. 4 and 5; Table 1). This is in agreement with the *in situ* hybridiza-

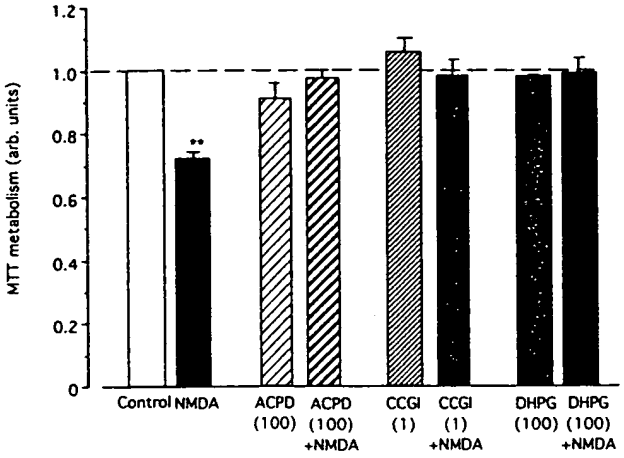

Fig. 7. Effects of mGluR agonists on the NMDA-induced toxicity in mesencephalic neuronal cultures. Neurons were pretreated for 5 min with ACPD (100 μM), CCGI (1 μM), or DHPG (100 μM) and then exposed to NMDA (100 μM) for 10 min. Data (absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of two or three independent experiments performed in quadruplicate. **, $p < 0.01$ in a one-tailed t test for NMDA values, compared with controls.

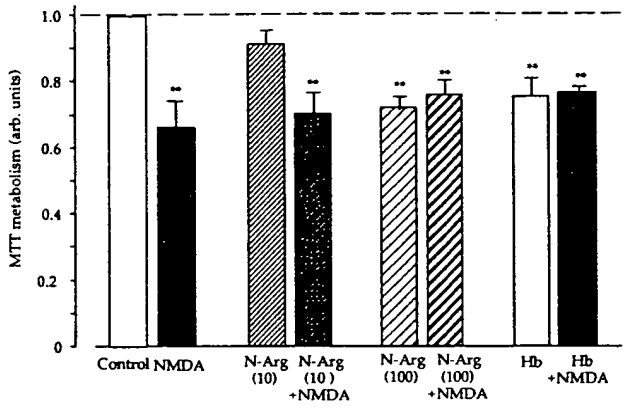

Fig. 8. Effect of NO deprivation on NMDA-induced toxicity in mesencephalic neuronal cultures. Neurons were pretreated for 5 min with N^G-nitro-L-arginine (*N-Arg*) (10 or 100 μM) or reduced hemoglobin (*Hb*) (1 μM) and then exposed to NMDA (100 μM) for 10 min. Data (absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of at least three independent experiments performed in quadruplicate. **, $p < 0.01$ in a one-tailed t test, compared with controls.

tion evidence for the expression of multiple mGluR mRNAs in the substantia nigra (17). ACPD and Quis increased intracellular InsPs with similar potencies. Such results differ from those obtained in other cell preparations, where Quis stimulates InsP formation more potently than does ACPD (15). However, Quis may also act on AMPA/kainate receptors, and InsP values measured may be the result of complex intracellular interactions that remain to be investigated. ACPD- and Quis-induced InsP formation was partially reduced by the noncompetitive antagonist AP3 (Table 1). ACPD triggered InsP formation and reduced basal and forskolin-stimulated adenylate cyclase activities with similar EC_{50} values. Another compound, DHPG, has been very recently proposed to be highly selective for the PI-coupled receptors (38). Indeed, it increased InsP levels also in mesencephalic cultures (Fig. 4) but, in our cell preparation, it also affected cAMP levels (Fig. 5B). Therefore, ACPD and DHPG did not discriminate between the two second messenger pathways, and it was not possible to determine, from their actions, which mGluR was responsible for NMDA receptor inhibition. In mesencephalic neurons, PI-linked receptors potentiate NMDA receptor activity, via protein kinase C (23), and a similar effect has been demonstrated in the hippocampus (39). Hence, we expected that activation of PI-linked mGluRs would produce a potentiation and not a block of NMDA-induced cGMP formation. The possibility that such an event occurred in our cell preparation cannot be excluded, but it was probably restricted to a small percentage of neurons and masked by the other prominent effect of negative modulation of NMDA responses.

CCGI was proposed to be selective for mGluR2 and mGluR3 expressed in a non-neuronal cell line, with an EC_{50} that was >1 order of magnitude lower than that for its effects on mGluR1, mGluR4, or mGluR5 (14, 26). In our neuronal preparation, this compound reduced basal and forskolin-stimulated cAMP levels at very low doses (maximal effect at approximately 1–5 μM). We consider it relevant that, at up to 10 μM, CCGI did not stimulate PI turnover but completely prevented NMDA receptor activation. These results strongly suggest that the mGluR responsible for the blockade of NMDA receptors observed herein is a subtype negatively coupled to adenylate cyclase. According to its pharmacology (14, 15), with CCGI being almost 2 orders of magnitude more potent than ACPD, and considering the *in situ* hybridization evidence reported in the literature (17), this receptor is likely the mGluR3 subtype.

The ability of mGluRs to modulate cell excitability through voltage-operated channels has been demonstrated in neurons from several brain areas (16). In mesencephalic neurons, the ACPD modulation of NMDA receptor activity was prevented by Ba^{2+} (Fig. 3). Although this ion does not allow identification of the class of K^+ channels involved, a possible explanation for this result is that ACPD elicited an outward current that hyperpolarized the cells, thus reducing the opening probability of the NMDA receptors, which undergo voltage-dependent Mg^{2+} block (12, 29). This would also be a possible explanation for why kainate receptors, which are voltage insensitive (12), were not influenced by ACPD or CCGI. The application of other K^+ channel blockers did not contribute further to the clarification of the mechanism(s) of such modulation. Indeed, we failed to mimic the action of Ba^{2+} with tetraethylammonium, 4-aminopyridine, or iberiotoxin, because by themselves they caused large increases in cGMP. However, other examples in the literature suggest that some K^+ conductances are particularly susceptible to block by external Ba^{2+} (28–30, 40), compared with other blockers. A contribution of neurotransmitter release appears unlikely, because of the presence of tetrodotoxin in the incubation buffer. In the substantia nigra pars compacta, other neurotransmitter receptors that are negatively linked to adenylate cyclase (dopamine D_2 and γ-aminobutyric acid type B receptors) activate outward K^+ currents and hyperpolarize dopaminergic neurons (28), causing also a decrease in NMDA receptor activity (29). The present study suggests that this could be a general mechanism (41) that is used also by

mGluR3 (or related mGluRs) prominently expressed in the substantia nigra pars reticulata (17).

The action of glutamate in the ventral mesencephalon is the object of increasing interest because of its contribution to human pathological processes, such as parkinsonism or psychoses (26). We used mesencephalic neuronal cultures as a model to study the early neurotoxic events induced by glutamate in this brain area. Here we show that 10-min treatment of mesencephalic neurons with NMDA induced mitochondrial damage that was already appreciable after 1 hr (Fig. 6). Pretreatment of mesencephalic neurons with ACPD (100 μM), CCGI (1 μM), or DHPG (100 μM) completely prevented NMDA-induced mitochondrial metabolic impairment (Fig. 7). It has been proposed that an important component of NMDA toxicity is due to NO release (33). The possibility that mGluR agonists exerted their neuroprotection via inhibition of NMDA-dependent NO formation was investigated. However, formation of this compound did not seem to be relevant for the manifestation of mitochondrial damage, at least under the neurotoxic conditions used in our study. Indeed, neither the NO synthase inhibitor N^G-nitro-L-arginine nor the removal of NO with reduced hemoglobin could prevent NMDA-mediated injury (Fig. 8), and other intracellular events must be considered (42).

To our knowledge, the data shown herein and the results of the electrophysiological study concerning the ACPD-mediated excitation of dopaminergic cells (13) are the only functional indications of mGluR existence in mesencephalic neurons. We think that our results can contribute to the understanding of the regulation of GluR subtypes in this brain region. Moreover, our study strongly encourages the development of compounds that are highly selective for mGluRs negatively linked to adenylate cyclase (as neuroprotective agents), which could have relevance also for human neuropathologies involving dysfunction of mesencephalic nuclei.

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Activation of Metabotropic Glutamate Receptors Protects Cultured Neurons Against Apoptosis Induced by β-Amyloid Peptide

Exhibit U (10/644,645)

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SUMMARY

Prolonged exposure of cultured cortical cells or cultured cerebellar granule cells to the residue 25–35 fragment of β-amyloid peptide (βAP), $\beta AP_{(25-35)}$, induced neuronal apoptosis, as revealed by morphological analysis, fluorescent chromatin staining, and immunodetection of oligonucleosomes released from the nucleus into the cytoplasm. $\beta AP_{(25-35)}$-induced apoptosis was insensitive to ionotropic glutamate receptor antagonists but was substantially attenuated by the metabotropic glutamate receptor (mGluR) agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid. The neuroprotective action of (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid was antagonized by (*RS*)-α-methyl-4-carboxyphenylglycine and was mimicked by (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine (a selective agonist of mGluR2 and -3 subtypes) and by L-2-amino-4-phosphobutanoate and L-serine-*O*-phosphate (selective agonists of mGluR4, -6, and -7 subtypes). However, whereas all of these drugs behaved as neuroprotectants in cultured cortical cells, only L-2-amino-4-phosphobutanoate and L-serine-*O*-phosphate [and not (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine] reduced $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells. The neuroprotective activity of mGluR agonists may be related to their ability to inhibit membrane Ca^{2+} conductance, because drugs that block voltage-sensitive Ca^{2+} channels, such as nimodipine or Co^{2+}, could also attenuate $\beta AP_{(25-35)}$-induced apoptosis.

βAP is a 40–42-amino acid peptide that accumulates as insoluble extracellular deposits in amyloid plaques of Alzheimer's brain (1, 2). The evidence that βAP aggregates are toxic to cultured neurons (3–8) supports the hypothesis that formation of amyloid plaques is causally linked to the cytoskeletal destabilization and neuronal degeneration that occur in Alzheimer's disease (9, 10). βAP, or its active, residue 25–35 fragment (8) $\beta AP_{(25-35)}$, destabilizes intracellular Ca^{2+} homeostasis (7, 11), thus amplifying the toxicity of NMDA receptor agonists or other excitotoxins (11, 12). A possible synergism between βAP and endogenous excitotoxins in Alzheimer's brain helps to explain the early onset of degeneration in the hippocampus (13, 14), where neurons are densely innervated by glutamatergic fibers. βAP, however, can also induce degeneration of cultured neurons through a different process, which does not incorporate an excitotoxic component. This process develops within 24–48 hr and exhibits the typical morphological and biochemical features of apoptosis, including formation of membrane blebs, fragmentation and condensation of chromatin, and DNA cleavage into oligonucleosomes (15, 16). Although it is uncertain to what extent βAP-induced apoptosis occurs *in vivo*, this process may be relevant for the degeneration of scattered neurons in Alzheimer's brain (9, 13, 17). Hence, the identification of pharmacological agents that oppose βAP-induced apoptosis may furnish new tools for the experimental therapy of Alzheimer's disease. We have focused on mGluR agonists, which are known to protect cultured neurons against excitotoxic damage (18–21). The activity of mGluR agonists has been compared with that of the VSCC blockers nimodipine and Co^{2+} (22–24), which have been reported to attenuate βAP-induced toxicity in cultured neurons (25).

ABBREVIATIONS: βAP, β-amyloid peptide; VSCC, voltage-sensitive Ca^{2+} channel(s); L-SOP, L-serine-*O*-phosphate; ACPD, 1-aminocyclopentane-1,3-dicarboxylic acid; AP4, L-2-amino-4-phosphonobutanoate; MCPG, (*RS*)-α-methyl-4-carboxyphenylglycine; DNQX, 6,7-dinitroquinoxaline-2,3-dione; MK-801, 10,11-dihydro-5-methyl-5*H*-dibenzo[*a*,*c*]cyclohepten-5,10-imine; DCG-IV, (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)-glycine; L-CCG-I, L-2-carboxycyclopropylglycine; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; MS, medium stock; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; GABA, γ-aminobutyric acid; DIV, days *in vitro*; ANOVA, analysis of variance; PLSD, protected least significant difference; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Experimental Procedures

Materials. βAP was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). NMDA, $CoCl_2$, cycloheximide, and L-SOP were obtained from Sigma Chemical Co. (St. Louis, MO). (1*S*,3*R*)-ACPD, quisqualate, L-AP4, MCPG, and DNQX were purchased from Tocris Neuramin (Essex, UK). MK-801 was obtained from Research Biochemicals (Natick, MA). DCG-IV and L-CCG-I were synthesized and kindly provided by Dr. H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Tokyo, Japan) and Dr. R. Pellicciari (Institute of Pharmaceutical Chemistry, University of Perugia, Perugia, Italy), respectively. Nimodipine was a generous gift from Bayer S.p.A. (Wuppertal, Germany).

Cell culture. Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–17 days of gestation, as described previously (26, 27). In brief, dissociated cortical cells were plated in 15-mm multiwell vessels (Falcon Primaria) on a layer of confluent glial cells (7–14 DIV), using, as a plating medium, Eagle's minimal essential medium (with Earle's salt, supplied glutamine-free; GIBCO) supplemented with 5% heat-inactivated horse serum, 5% fetal calf serum, 2 mM glutamine, and 21 mM glucose. After 3–5 DIV, proliferation of glial cells was halted by addition of 10 μM cytosine arabinoside. After an additional 3 days, cultures were shifted to a maintenance medium identical to the plating medium but lacking fetal calf serum (MS). Subsequent partial medium replacement was carried out twice each week. Cultures at 13–16 DIV were used.

Primary cultures of cerebellar granule cells were prepared from 8-day old rats, as described previously (28). In brief, dissociated cerebellar cells were suspended in basal Eagle's medium (GIBCO) containing 10% fetal calf serum, 2 mM glutamine, 0.05 mg/ml gentamicin, and 25 mM K^+ and were plated onto 35-mm Nunc Petri dishes that had been precoated with poly-L-lysine (10 μg/ml). Cytosine arabinoside (10 μM) was added to the cultures after 18 hr to inhibit proliferation of non-neuronal cells. Cultures at 7–9 DIV contained >90% granule cells, with about 3–5% GABAergic neurons and few glial and endothelial cells as contaminants (28, 29).

Experimental procedure and assessment of neuronal degeneration. $\beta AP_{(25\text{-}35)}$ was solubilized in sterile, doubly distilled water at an initial concentration of 2.5 mM and was stored frozen at −20°. Cortical cultures, shifted into MS, were exposed to βAP for different times (up to 48 hr) before the assessment of neuronal degeneration. mGluR agonists, VSCC blockers, or ionotropic glutamate receptor antagonists were all coapplied with βAP and maintained for the entire exposure time. To test the combined effect of βAP and an excitotoxic insult, we exposed cortical cultures to a submaximal concentration of $\beta AP_{(25\text{-}35)}$ (12.5 μM) for 24 hr. Cultures were then shifted into a HEPES-buffered salt solution containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, and 15 mM glucose, and NMDA (60 μM) was added for 10 min at room temperature. At the end of this incubation, cultures were returned to the MS and incubated for 20 hr before the assessment of neuronal degeneration. mGluR agonists were applied in combination with NMDA.

Cultured cerebellar granule cells at 5–6 DIV were shifted into a medium identical to the plating medium but lacking fetal calf serum. Three hours later, cultures were exposed to $\beta AP_{(25\text{-}35)}$, in the absence or presence of mGluR agonists and/or ionotropic glutamate receptor antagonists, for 48–72 hr. Neuronal degeneration was assessed by combining phase-contrast microscopy, fluorescent chromatin staining, photometric enzyme immunoassays for the determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), and determination of the activity of LDH released from damaged neurons into the medium.

Fluorescent staining of nuclear chromatin. Cultured cells were fixed with methanol/acetic acid (3:1) for 30 min, washed three times in phosphate-buffered saline, and then incubated for 15 min at 37° with 0.4–0.8 μg/ml levels of the fluorescent nuclear dye Hoechst 33258. After a two-step wash with water, cells were viewed for nuclear chromatin morphology with a fluorescence microscope, using an oil-immersion objective. Apoptotic neurons were recognized by nuclear condensation and/or fragmented chromatin. In the phase-contrast analysis, apoptotic neurons were irregularly shaped, with shrunken cell bodies and/or distrophic neurites. The number of viable and apoptotic neurons was counted in three fixed fields/culture dish.

Immunodetection of oligonucleosomes. Mono- and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic

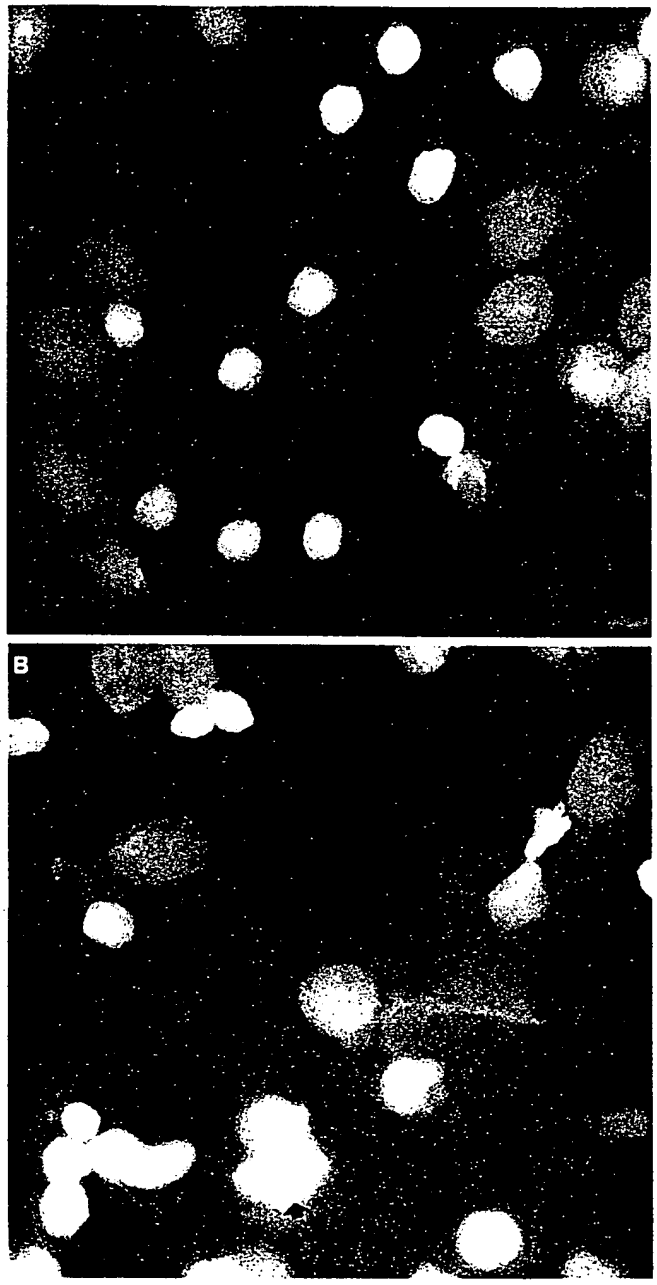

Fig. 1. Fluorescent chromatin staining of cultured cortical cells exposed for 48 hr to $\beta AP_{(25\text{-}35)}$ (25 μM). A, Control cells with regularly dispersed chromatin; B, examples of fragmentation (*arrows*) or condensation (*arrowheads*) of nuclear chromatin in neurons surrounded by aggregates of $\beta AP_{(25\text{-}35)}$ (*). Note that the larger nuclei of astrocytes do not show any morphological alteration of chromatin in cultures treated with $\beta AP_{(25\text{-}35)}$. In both A and B the incubation medium contained MK-801 (10 μM) and DNQX (30 μM).

neurons were detected by using a sandwich ELISA (Cell Death Detection ELISA; Boehringer Mannheim, Germany). The assay is based on the quantitative sandwich ELISA principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. For sample preparation, cortical cultures were washed in phosphate-buffered saline and then scraped into 400 μl of cold buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol, pH 7.9. Cells were placed on ice for 10 min, added to Nonidet P-40 (0.1% final concentration), and vortex-mixed for 10 sec. Individual homogenates were centrifuged for 10 min with an Eppendorf microfuge at maximal speed. The supernatant was diluted to yield 13 × 10^3 cell equivalents/ml and used for immunodetection. The assay was performed as follows: (i) an antibody that reacts with the histones H1, H2A, H2B, H3, and H4 was fixed on the wall of a microtiter plate module provided with the kit; (ii) samples prepared as described above were added to the plate containing the immobilized anti-histone antibody; (iii) anti-DNA monoclonal antibodies conjugated to peroxidase were added, to allow their binding to the DNA part of nucleosomes; and (iv) after removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) as a substrate.

Measurement of LDH activity released from damaged cells into the medium. LDH activity was measured spectrophotometrically, as described previously (30).

Results

Toxicity of $\beta AP_{(25-35)}$ in cultured cortical neurons. The occurrence of apoptotic degeneration in cultured cortical neurons exposed to $\beta AP_{(25-35)}$ has been recently shown by DNA laddering and electron microscopic analysis (15, 16). To demonstrate the same phenomenon in mixed cortical cultures, we combined phase-contrast microscopy, nuclear chromatin staining with the fluorescent dye Hoechst 33258, and an ELISA that allows the detection of oligonucleosomes released from the nucleus into the cytoplasm. The latter method has been used for the assessment of the neuroprotective activity of mGluR agonists. Phase-contrast analysis of

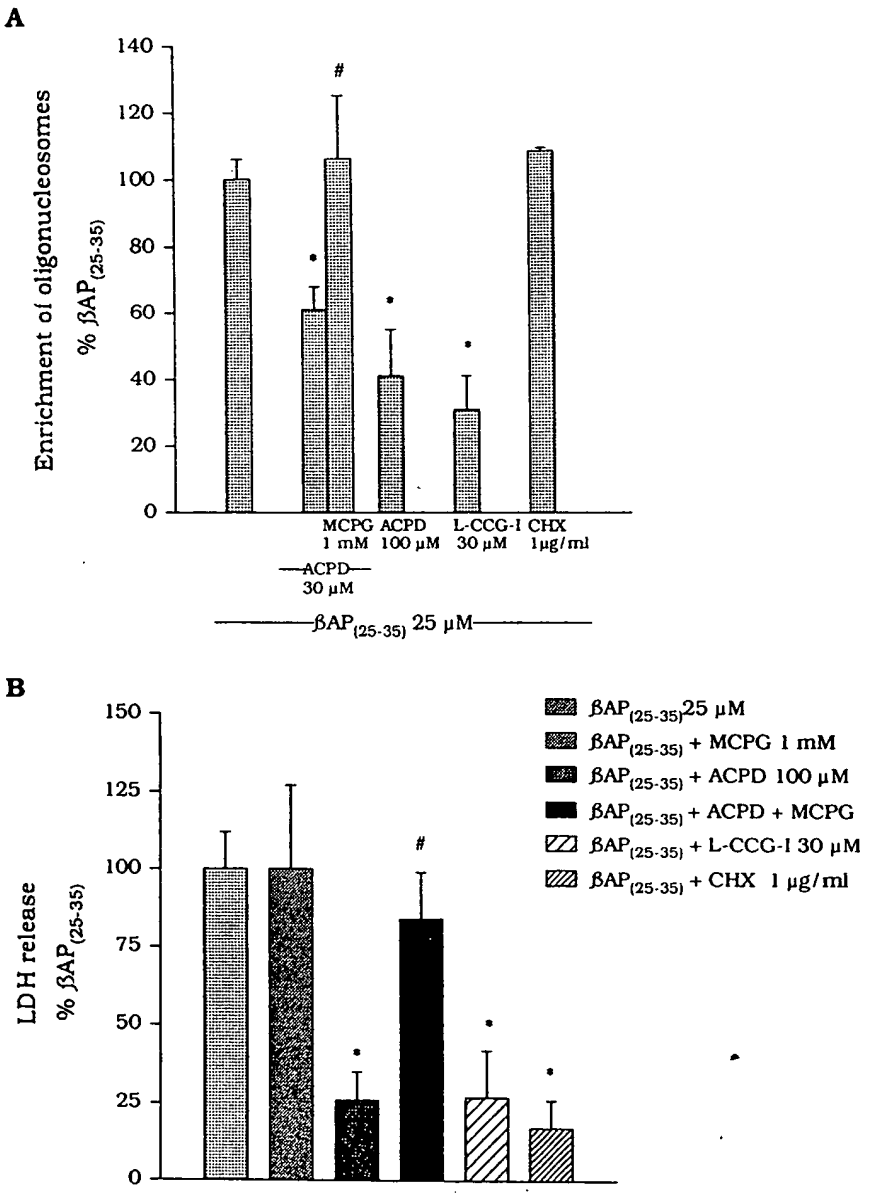

Fig. 2. A, Immunodetection of oligonucleosomes in cultures exposed for 48 hr to $\beta AP_{(25-35)}$ (25 μM), in the absence or presence of mGluR agonists or antagonists or cycloheximide (*CHX*). B, Measurement of LDH release under the same conditions as in A. In A, values (means ± standard errors) are expressed as percentages of the $\beta AP_{(25-35)}$ effect and were calculated from six to 28 determinations in two to four individual experiments. In a typical experiment, absolute values were 734 ± 58 and 1326 ± 84 milliunits of absorbance (A_{405nm}/A_{490})/1300 cell equivalents (six determinations) in control cultures and in cultures treated with βAP(25-35), respectively. Cycloheximide applied for 48 hr in control cultures induced a nonsignificant increase in oligonucleosome release, which was 32 ± 12% of the value observed with $\beta AP_{(25-35)}$ (12 determinations). In B, values (means ± standard errors) are expressed as percentages of the $\beta AP_{(25-35)}$ effect and were determined in medium collected from parallel cultures treated as in A. The medium was collected 48 hr after the addition of $\beta AP_{(25-35)}$. All experiments were performed in the presence of 10 μM MK-801 and 30 μM DNQX. Basal LDH values ranged from 20 to 75 milliunits of absorbance/min/well in most experiments. In two experiments, however, basal LDH values were <10 milliunits of absorbance/min/well. Stimulation of LDH release by $\beta AP_{(25-35)}$ ranged from about 90 to 180% above basal levels in different experiments. (1S,3*R*)-ACPD, L-CCG-I, and cycloheximide (all applied for 48 hr) did not affect the basal LDH activity in cultures that were not treated with $\beta AP_{(25-35)}$. Values of LDH activity were 88 ± 7, 95 ± 3, and 104 ± 11% of control in response to (1S,3*R*)-ACPD (100 μM), L-CCG-I (100 μM), and cycloheximide (1 μg/ml), respectively (six to 18 determinations). * and #, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with βAP alone (*) or βAP plus 30 μM (1S,3*R*)-ACPD (#).

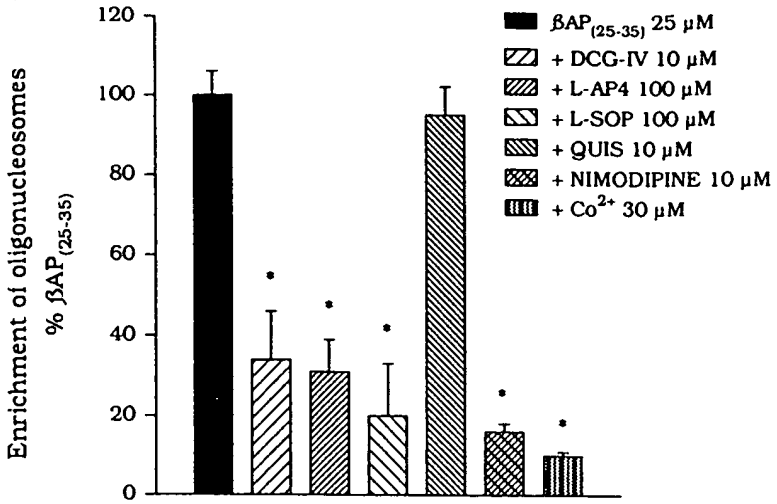

Fig. 3. Attenuation of $\beta AP_{(25-35)}$-induced apoptosis in cultured cortical cells by agonists of class II or III mGluRs or inhibitors of VSCC. All drugs were coincubated with $\beta AP_{(25-35)}$ and 10 μM MK-801 plus 30 μM DNQX. Values are expressed as in Fig. 2 and were calculated from six to 12 individual determinations (two independent experiments). *, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with $\beta AP_{(25-35)}$ alone. *QUIS*, quisqualate.

TABLE 1

Necrotic degeneration induced by the combination of $\beta AP_{(25-35)}$ and NMDA, in the absence or presence of mGluR agonists

For estimation of LDH activity, the incubation medium was collected 20 hr after the NMDA pulse. $\beta AP_{(25-35)}$ was applied to the cultures 24 hr before NMDA. Values are means ± standard errors of six determinations.

	LDH release
	milliunits of absorbance/min/well
Control	4.5 ± 3.7
$\beta AP_{(25-35)}$ (12.5 μM)	21 ± 2
NMDA (60 μM)	37 ± 6.8
$\beta AP_{(25-35)}$ + NMDA	66 ± 2
$\beta AP_{(25-35)}$ + NMDA + DCG-IV (5 μM)	72 ± 15
$\beta AP_{(25-35)}$ + NMDA + L-SOP (100 μM)	87 ± 22

cortical cultures exposed to $\beta AP_{(25-35)}$ (25 μM) showed neurons with shrunken and irregularly shaped cell bodies and degenerating neurites. No neuronal swelling was observed throughout the incubation with $\beta AP_{(25-35)}$ (data not shown). Astrocytes did not show any morphological sign of toxicity (see also Ref. 16). Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in neurons of cultures treated with $\beta AP_{(25-35)}$. Chromatin staining of astrocytes did not differ between control and $\beta AP_{(25-35)}$-treated cultures (Fig. 1). All of these morphological features typical of apoptotic degeneration developed within 24–48 hr of incubation with $\beta AP_{(25-35)}$ and were not influenced by the presence of the ionotropic glutamate receptor antagonists MK-801 (10 μM) and DNQX (30 μM) (data not shown). The amount of oligonucleosomes released from the nucleus into the cytoplasm was almost doubled in cultures exposed to $\beta AP_{(25-35)}$ (see the legend to Fig. 2A), and substantial increases in LDH release were observed only with incubation times of >24 hr, when cell lysis was completed (Fig. 2B; see also Ref. 16). In agreement with previous results (16), the protein synthesis inhibitor cycloheximide (1 μg/ml) delayed the lysis of cells exposed to $\beta AP_{(25-35)}$, as reflected by a reduced release of LDH at 48 hr after addition of the peptide (Fig. 2B). However, cycloheximide did not reduce the amount of oligonucleosomes detected by the ELISA in cultures exposed to $\beta AP_{(25-35)}$ (Fig. 2A).

To study the influence of mGluR activation on βAP-induced toxicity, we coapplied $\beta AP_{(25-35)}$ with mGluR agonists in the presence of 10 μM MK-801 and 30 μM DNQX (added to avoid any secondary activation of ionotropic glutamate receptors). The mixed mGluR agonist (1*S*,3*R*)-ACPD attenuated $\beta AP_{(25-35)}$-induced apoptosis without preventing the formation of βAP aggregates in cultures (data not shown). The neuroprotective action of (1*S*,3*R*)-ACPD was antagonized by MCPG [coapplied with (1*S*,3*R*)-ACPD and $\beta AP_{(25-35)}$] and mimicked by L-CCG-I (Fig. 2), which also behaves as a mixed mGluR agonist (31). Neither (1*S*,3*R*)-ACPD nor L-CCG-I, when applied for 48 hr, attenuated spontaneous neuronal degeneration in control cultures (see the legend to Fig. 2).

DCG-IV (10 μM), L-AP4 (100 μM), and L-SOP (100 μM), which selectively activate mGluR subtypes negatively linked to adenylyl cyclase (32–38), protected cortical neurons against $\beta AP_{(25-35)}$-induced apoptosis, whereas quisqualate (10 μM) was inactive (Fig. 3). The protective action of mGluR agonists against apoptosis was mimicked by drugs that inhibit VSCC, such as Co^{2+} (30 μM) or nimodipine (10 μM) (Fig. 3).

We also studied the influence of mGluR agonists on toxicity induced by a 10-min pulse with NMDA in cultures exposed for 24 hr to 12.5 μM $\beta AP_{(25-35)}$. This combination resulted in extensive necrotic degeneration, characterized by initial neuronal swelling and LDH release, which was substantial at 2–5 hr after the NMDA pulse. Neither DCG-IV nor L-SOP protected cortical neurons against toxicity induced by the combination of $\beta AP_{(25-35)}$ and NMDA (Table 1).

Toxicity of $\beta AP_{(25-35)}$ in cultured cerebellar granule cells. $\beta AP_{(25-35)}$ also induced apoptosis when added to primary cultures of cerebellar granule cells, although the number of apoptotic neurons after 48 hr of exposure was smaller than that in cultures of cortical neurons. The high level of homogeneity of cultured cerebellar granule cells and the large cell nuclei allowed a reliable microscopic count of neurons bearing chromatin fragmentation or condensation (Fig. 4). $\beta AP_{(25-35)}$-induced apoptosis in cerebellar granule cells was insensitive to MK-801 (10 μM) and DNQX (30 μM) (data not shown), which were therefore routinely included in the incubation medium.

Addition of (1*S*,3*R*)-ACPD [200 μM, coapplied with $\beta AP_{(25-35)}$] attenuated $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar

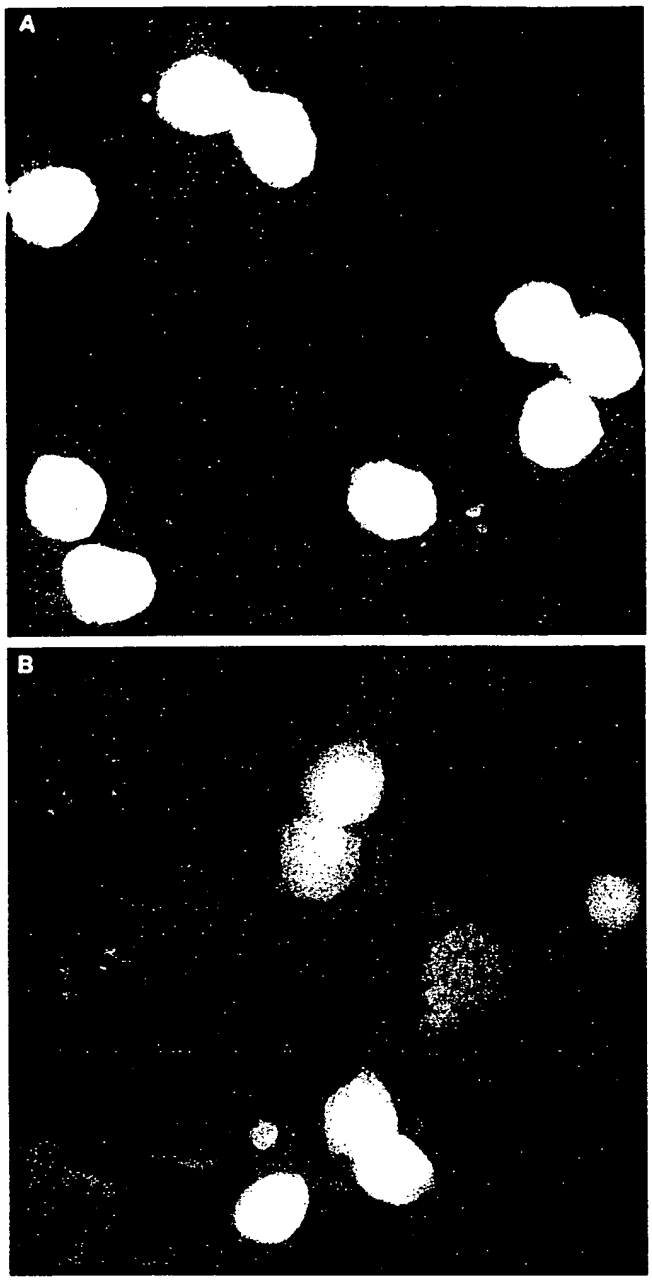

Fig. 4. Nuclear chromatin staining of cultured cerebellar granule cells exposed for 48 hr to 25 μM $\beta AP_{(25-35)}$. A, Control granule cells with regularly dispersed nuclear chromatin; B, examples of chromatin fragmentation (*arrow*) or condensation (*arrowheads*) in granule cells surrounded by aggregates of $\beta AP_{(25-35)}$ (*). In both A and B, the incubation medium contained MK-801 (10 μM) and DNQX (30 μM).

granule cells. The action of (1*S*,3*R*)-ACPD was mimicked by L-AP4 or L-SOP (both at 100 μM), whereas DCG-IV (10 μM) was inactive (Fig. 5).

Discussion

The evidence that aggregates of βAP or its active fragment, $\beta AP_{(25-35)}$, are toxic to cultured neurons has encouraged the search for drugs that act as specific neuroprotective agents in Alzheimer's disease. When βAP is applied to cultures in combination with an excitotoxin, neuronal toxicity progresses rapidly, with the typical features of necrotic degeneration (neuronal swelling and lysis of cell membranes, accompanied by an early leakage of LDH into the medium). This process is sensitive to ionotropic glutamate receptor antagonists (11, 12). When βAP is applied to cultures in the absence of an excitotoxic insult, toxicity has a slow progression and neuronal degeneration follows an apoptotic pathway (15, 16). Electron microscopic analysis of cultured cortical neurons exposed to βAP or to $\beta AP_{(25-35)}$ shows spheriform protrusion (or "blebs") of the plasma membrane, as well as fragmentation and condensation of nuclear chromatin (16), all features that are typical of apoptotic degeneration (39). DNA analysis by agarose gel electrophoresis reveals the presence of oligonucleosome-size fragments (16), which are a hallmark of apoptosis (39). βAP-induced apoptosis is preceded by a substantial increase in c-*jun* mRNA (40) and is reported to be attenuated by inhibition of protein synthesis with cycloheximide (16). This suggests that some of the intracellular events that are involved in βAP-induced apoptosis require the synthesis of specific proteins. βAP-induced apoptosis is resistant to ionotropic glutamate receptor antagonists (Ref. 16 and present data) and, in Alzheimer's brain, may provide an insidious mechanism whereby βAP present in amyloid plaques promotes the degeneration of scattered neurons regardless of their innervation. The identification of pharmacological agents that attenuate βAP-induced apoptosis may therefore have important implications for the experimental therapy of Alzheimer's disease. Knowing that mGluR agonists protect cultured neurons against excitotoxic degeneration (18–21), we tested their efficacy in mixed cortical cultures exposed to $\beta AP_{(25-35)}$. This model was selected because it has been widely used for the evaluation of neuronal toxicity and maintains the physiological interplay between neurons and astrocytes. The occurrence of neuronal apoptosis in mixed cortical cultures exposed to $\beta AP_{(25-35)}$ was indicated by the following observations: (i) the absolute lack of neuronal swelling at any stage of the degenerative process; (ii) the presence of chromatin fragmentation and condensation, revealed by the fluorescent dye Hoechst 33258; (iii) the lack of temporal correlation between morphological abnormalities and LDH release, which suggests that membrane integrity is preserved until the late stages of neuronal degeneration; and (iv) the immunodetection of an increased amount of oligonucleosomes released from the nucleus into the cytoplasm. We also studied the toxic effects of $\beta AP_{(25-35)}$ in the presence of cycloheximide, because new protein synthesis is generally required for the induction of apoptosis, although there are numerous cases in which protein synthesis inhibitors do not protect against apoptotic death (41). Addition of cycloheximide reduced the extent of cell lysis after 48-hr exposure to $\beta AP_{(25-35)}$. However, cycloheximide did not reduce the formation of oligonucleosomes, a process that is generally considered as an early marker of apoptotic death. One possible explanation is that protein synthesis is not required for chromatin fragmentation but is involved in one of the processes that lead to cell shrinkage and eventually lysis in response to βAP.

To study the influence of mGluR activation on $\beta AP_{(25-35)}$-induced apoptosis, we initially used the selective mGluR agonist (1*S*,3*R*)-ACPD, the active isomer of *trans*-ACPD. ACPD is known to protect cultured neurons against excito-

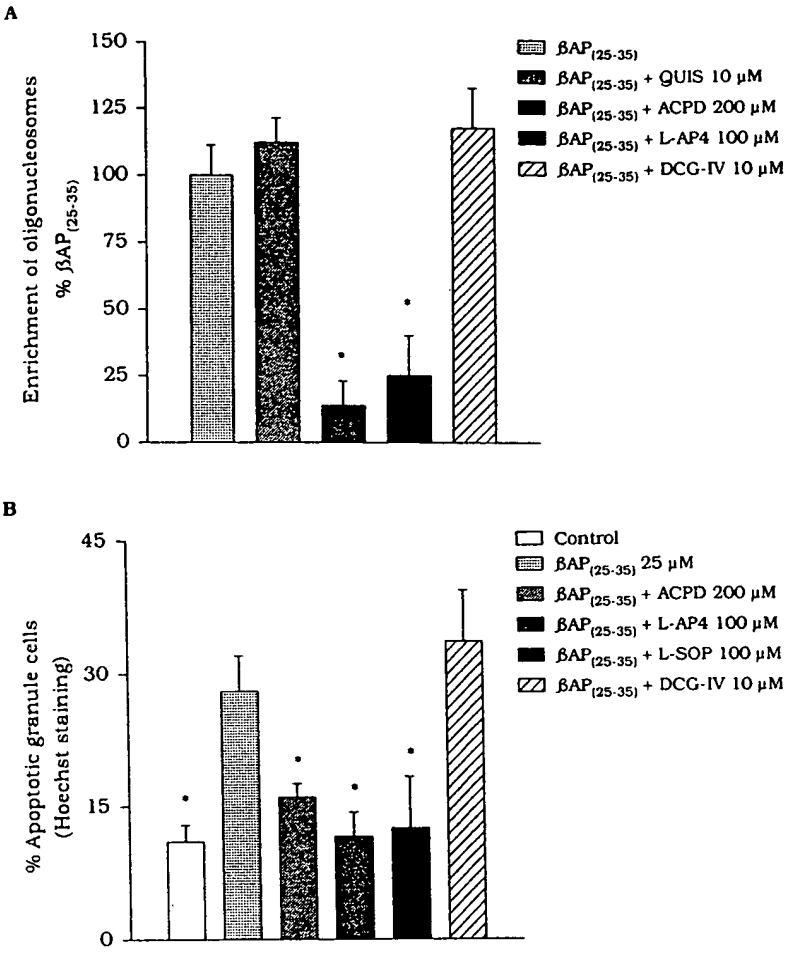

Fig. 5. $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells treated with mGluR agonists. All drugs were coapplied with $\beta AP_{(25-35)}$ in the presence of MK-801 (10 μM) and DNQX (30 μM). The extent of apoptotic degeneration was quantitated either by detecting, by ELISA, the amount of oligonucleosomes released into the cytoplasm (A) or by counting the number of granule cells bearing chromatin abnormalities (B). Values are means ± standard errors of three to six individual determinations. *, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with βAP alone. Basal and $\beta AP_{(25-35)}$-induced values in A were 75 ± 6 and 138 ± 16 milliunits of absorbance (A_{405nm}/A_{490nm})/1300 cell equivalents, respectively. *QUIS*, quisqualate.

toxic degeneration (20, 21), although it can also induce neuronal toxicity when locally infused into the rat hippocampus or corpus striatum (42–44). (1*S*,3*R*)-ACPD applied in combination with $\beta AP_{(25-35)}$ attenuated neuronal apoptosis, and its action was prevented by the mGluR antagonist MCPG (45). mGluRs form a family of at least seven subtypes, which, in transfected cells, are either coupled to polyphosphoinositide hydrolysis (mGluR1 and -5, class I) (31, 46, 47) or negatively linked to adenylyl cyclase (mGluR2 and -3, class II, and mGluR4, -6, and -7, class III) (31, 35–38). All of these subtypes are activated by (1*S*,3*R*)-ACPD (31). We therefore wondered which subtype or class of subtypes mediates the neuroprotective action of (1*S*,3*R*)-ACPD against βAP-induced apoptosis. The drugs we used to address this question were the following: (i) DCG-IV, which is highly selective for mGluR2 and -3 subtypes (32–34) and at high concentrations can also activate NMDA receptors (33), an action that was prevented by the inclusion of MK-801 in the incubation medium; (ii) L-CCG-I, which behaves as a mixed mGluR agonist but with preferential activity at mGluR2 and -3 subtypes (31); (iii) L-AP4 and L-SOP, which selectively activate mGlu4, -6, and -7 subtypes (36–38); and (iv) quisqualate, which, at the concentration we used (10 μM), activates mGluR1 and -5, with little or no activity at the other subtypes (35, 37).

In mixed cultures of cortical cells, quisqualate, L-CCG-I, or (1*S*,3*R*)-ACPD, but not DCG-IV, L-AP4, or L-SOP, stimulates polyphosphoinositide hydrolysis (21).[1] DCG-IV, L-CCG-I, L-AP4, L-SOP, and (1*S*,3*R*)-ACPD inhibit forskolin-stimulated cAMP formation in pure cortical neuronal cultures,[1] whereas in mixed cultures their effects are masked by the large response of astrocytes to forskolin (21).[1]

DCG-IV, L-CCG-I, L-AP4, or L-SOP, but not quisqualate, mimicked the protective action of (1*S*,3*R*)-ACPD against $\beta AP_{(25-35)}$-induced neuronal apoptosis in mixed cortical cultures. These results confirm the recent view that activation of class II or III mGluR subtypes protects cortical neurons against excitotoxic death (21, 48)[1] and that DCG-IV attenuates neuronal apoptosis induced by staurosporine or glucose deprivation (48). Neuroprotection, however, is limited by the strength of the toxic insult, because neither DCG-IV nor L-SOP attenuated neuronal toxicity induced by a combination of $\beta AP_{(25-35)}$ and NMDA, a condition that resulted in rapid and intense necrotic degeneration of cultured cortical neurons.

To establish whether the neuroprotective influence of mGluRs against βAP-induced apoptosis could be extended to other neuronal populations, we used cultured cerebellar granule cells, a model that is often utilized for the screening

[1] V. Bruno, G. Battaglia, A. Copani, R. G. Giffard, G. Raciti, G. Raffaele, H. Shinozaki, and F. Nicoletti. Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. Submitted for publication.

of neuroprotective agents (19, 49, 50) and has been shown to be sensitive to the toxic effects of $\beta AP_{(25-35)}$ (51). Cultured cerebellar granule cells represent a homogeneous population of glutamatergic neurons, which express at least five mGluR subtypes (mGluR1–5), although mGluR1 and -4 predominate over the others (52). Under our experimental conditions (cultures grown in the presence of 25 mM K^+ and shifted into serum-free medium at 5 DIV), $\beta AP_{(25-35)}$ induced apoptosis when applied to cultured cerebellar granule cells in the presence of ionotropic glutamate receptor antagonists. However, only 30% of the total cell population underwent apoptosis in response to $\beta AP_{(25-35)}$. This reflects an intrinsic resistance of cultured cerebellar granule cells, which is also reflected by the lower spontaneous apoptosis, compared with cortical cultures (compare the control values for oligonucleosomes given in the legends to Figs. 2A and 5A). $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells was attenuated by (1*S*,3*R*)-ACPD or by the mGluR4/6/7 agonists L-AP4 and L-SOP but not by quisqualate or by the mGluR2/3 agonist DCG-IV. This is consistent with the evidence that cerebellar granule cells express large amounts of mGluR4 mRNA both in culture (52) and in the intact cerebellum (35), whereas mGluR2 and -3 mRNAs are expressed to a much lower extent.

Although class II or III mGluR subtypes are negatively linked to adenylyl cyclase (31, 35–38), this mechanism is difficult to reconcile with the evidence that cAMP itself is neuroprotective (53). Both L-AP4 and mGluR2 agonists inhibit Ca^{2+} influx through VSCC in cultured neurons (23, 24). Inhibition of VSCC may contribute to the neuroprotective effect of mGluR agonists, because βAP is known to increase intracellular free Ca^{2+} concentrations in cultured neurons (7, 11). It is consistent with this hypothesis that drugs that inhibit VSCC, such as Co^{2+} or nimodipine, attenuate the toxic action of βAP (25) and here reduced the extent of $\beta AP_{(25-35)}$-induced apoptosis. In conclusion, the present results provide additional evidence for the existence of "neuroprotective" mGluR subtypes, which may therefore be potential targets for specific drugs in the experimental therapy of acute or chronic neurodegenerative disorders.

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Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence

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Exhibit V (10/644,645)

1 The contribution of various excitatory amino acid (EAA) receptors (NMDA, AMPA/kainate and metabotropic) in the brain to the development of morphine dependence was examined. This was performed by measuring the severity of the precipitated withdrawal syndrome following chronic subcutaneous (s.c.) morphine and intracerebroventricular (i.c.v.) EAA antagonist treatment.

2 Continuous subcutaneous (s.c.) treatment with morphine sulphate (36.65 μmol day^{-1}) produced an intense and reliable naloxone-precipitated withdrawal syndrome.

3 Chronic i.c.v. treatment with antagonists selective for metabotropic and NMDA receptors, but not AMPA/kainate receptors, significantly attenuated abstinence symptoms. Conversely, EAA antagonists had very little effect on non-withdrawal behaviours.

4 These results suggest that, as well as changes elicited by activation of NMDA receptors, metabotropic receptors and intracellular changes in the phosphatidylinositol (PI) second-messenger system or the cyclic adenosine 3',5'-monophosphate (cAMP) second messenger system, to which EAA metabotropic receptors are linked, may be involved in the development of opioid dependence with chronic morphine treatment.

Keywords: Opioid; morphine; dependence; glutamate; metabotropic glutamate receptor; AMPA; kainate; NMDA; abstinence syndrome; excitatory amino acids

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Introduction

Although opioid drugs such as morphine are widely used for the management of pain, their clinical usefulness is limited by the development of tolerance and dependence that occurs with their chronic use. Tolerance is indicated by a decreased efficacy of the drug with repeated administration, and results in a need to increase the morphine dose in order to achieve the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, and leads to an aversive withdrawal or abstinence syndrome when morphine administration is terminated. Recently, it has been demonstrated that co-administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists attenuates the development of morphine tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). Since the endogenous excitatory amino acid (EAA) glutamate activates NMDA receptors, it is likely that glutamate contributes to the development of these phenomena. In addition to NMDA receptors, glutamate acts at at least two other types of ionotropic receptors: receptors at which α-2-amino-3-(hydroxy-5-methylisoxazol-4yl)propanoic acid (AMPA) is a selective agonist and receptors at which kainate is a selective agonist; and metabotropic receptors (Mayer & Westbrook, 1987a; Monaghan *et al.*, 1989). While a role for the NMDA receptor has already been suggested, the specific contribution of these other receptors to behavioural indices of opioid tolerance and dependence has not been investigated.

The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^+ (MacDermott *et al.*, 1986; Mayer *et al.*, 1987) and is gated in a voltage-dependent fashion by Mg^{2+} (Mayer *et al.*, 1984; Nowak *et al.*, 1984). It is the voltage-dependent Ca^{2+} permeability of the NMDA receptor that is thought to be necessary for use-dependent synaptic plasticity (Cotman *et al.*, 1988) and may be critical for the development of neuronal changes that mediate opioid tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). AMPA and kainate receptors gate cation channels that are permeable to Na^+, but for the most part have negligible permeability to Ca^{2+} (Mayer & Westbrook, 1987b; Murphy & Miller, 1989). However, AMPA/kainate receptors that exhibit Ca^{2+} permeability have recently been cloned (Miller, 1991; Sommer & Seeburg, 1992). Traditionally, AMPA/kainate receptors are thought to be involved in the mediation of rapid excitatory responses to EAA transmitters (Kiskin *et al.*, 1986; Jonas & Sakmann, 1992) and may contribute to neuronal plasticity by relieving the NMDA receptor of its voltage-dependent block by Mg^{2+}. Unlike ionotropic receptors, metabotropic glutamate receptors are not linked to cation channels. Instead they are coupled directly to the cell membrane by a G protein (Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987). Several subtypes of metabotropic glutamate receptors have recently been cloned. Some subtypes affect phosphatidylinositol (PI) hydrolysis (mGluR1α, mGluR1β and mGluR5), while others affect the production of adenosine 3',5'-cyclic monophosphate (cAMP) (mGluR2, mGluR3, mGluR4) (Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the PI system activates phospholipase C, which catalyses phosphatidylinositol hydrolysis, leading to the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the cAMP second messenger system generally leads to decreased production of cAMP, although activation of mGluR1α stimulates an increase in cAMP (Schoepp & Conn, 1993). Through the increased production of intracellular messengers associated with PI hydrolysis, or decreased production of cAMP, metabotropic receptor activation may play an important role in the long-term effects mediated by glutamate (Nicoletti *et al.*, 1991), and like NMDA receptors may be critical to the development of neuronal changes mediating opioid tolerance and dependence.

In the present study we have investigated the contribution of various EAA receptor subtypes in the brain to the

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development of opioid dependence. This purpose was achieved by examining the effects of the intracerebroventricular (i.c.v.) administration of selective EAA receptor antagonists concurrently with the chronic subcutaneous (s.c.) administration of morphine. NMDA receptors were antagonized with the non-competitive antagonist 5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine hydrogen maleate (MK-801) (Wong *et al.*, 1986); AMPA/kainate receptors were antagonized with 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine-hydrochloride (GYKI 52466) (Donevan & Rogawski, 1993). Although distinct families of high-affinity AMPA and kainate receptors have been isolated, the functional distinction between these receptors is not entirely clear (Barnard & Henley, 1990). Consequently, pharmacological investigations with receptor antagonists are limited to investigations of non-selective AMPA/kainate receptor effects. Metabotropic receptors were antagonized with the highly selective antagonist (*S*)-4-carboxyphenylglycine [(*S*)-4C-PG] (Birse *et al.*, 1993; Eaton *et al.*, 1993) and a more commonly used, yet less selective antagonist, L-2-amino-3-phosphonopropanoic acid (L-AP3) (Schoepp *et al.*, 1990; Birse *et al.*, 1993).

Methods

Subjects and surgery

Subjects were male Long Evans rats (280–350 g). The rats were housed 2–3 per cage, on a 12:12 h light–dark cycle (lights on at 06:00 h), with food and water available *ad libitum*.

On day 0 rats were anaesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, 60 mg kg^{-1}), and 23 gauge stainless steel cannulae, attached to model 2001 Alzet osmotic mini-pumps filled with one of the EAA antagonist solutions or saline, were implanted stereotaxically in the lateral ventrical (AP = −1.3 mm and L = −1.8 mm from bregma and V = −3.8 mm from the top of the skull; Paxinos & Watson, 1986). While the rats were still under pentobarbital anaesthesia, one unprimed (i.e. not yet pumping) model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. These morphine-containing pumps started pumping the morphine solution approximately 2–4 h following implantation. On the following day, day 1, rats were briefly anaesthetized with halothane and a second unprimed model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. This two day pump implantation procedure was used to reduce the risk of mortality resulting from the accumulation of lethal systemic morphine concentrations prior to any tolerance development. To assess the effects of chronic i.c.v. EAA antagonist treatment on behaviour in rats not dependent on morphine, some rats were given vehicle or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 without concurrent morphine treatment.

Drugs

MK-801, L-AP3 and GYKI 52466 were obtained from Research Biochemicals, (Natick, MA, U.S.A.), while (*S*)-4C-PG was purchased from Tocris Neuramin (Bristol, U.K.). EAA antagonists were continuously infused at a rate of 1 µl h^{-1} in the following concentrations: 1.6 nmol day^{-1}, 8 nmol day^{-1} and 40 nmol day^{-1}. Morphine sulphate (Sabex, Montreal, Canada) was continuously delivered at a rate of 10 µl h^{-1} for a total dose of 36.65 µmol day^{-1} morphine sulphate.

Withdrawal measurement

Precipitated abstinence symptoms were assessed on the seventh day of treatment (while all pumps were still delivering antagonists and morphine) after injection of naloxone (1 mg kg^{-1} s.c.). For 10 min before and 40 min after naloxone injection, the withdrawal symptoms were assessed by measuring the amount of time spent teeth chattering and writhing, as well as by counting jumps and wet dog shakes (Bläsig *et al.*, 1973). The time spent in non-withdrawal behaviours (ambulating, rearing, grooming and resting) was also measured for comparison, for 10 min before and after the injection of naloxone, in rats treated with i.c.v. EAA antagonists either alone or with s.c. morphine.

Statistical analysis

Timed withdrawal behaviours (teeth chattering, writhing) were analysed using ANOVA, followed by *post hoc* tests on significant main effects. Counted withdrawal behaviours (number of jumps and wet dog shakes) were analysed using the Kruskal–Wallis ANOVA for non-parametric data, followed by Mann–Whitney *U*-tests on significant main effects.

The effect of EAA antagonist treatment on non-withdrawal behaviours (ambulating, rearing, grooming and resting) was assessed by comparing the first two time blocks (i.e. 10 min prior to naloxone injection and 10 min after naloxone injection) for rats in each treatment group. Planned comparisons were used to analyse differences in the proportion of time spent in each timed non-withdrawal and the two timed withdrawal behaviours during these two time blocks across the different treatment conditions.

Results

Administration of 36.65 µmol day^{-1} s.c. morphine sulphate by Alzet pump produced an intense and reliable naloxone precipitated abstinence syndrome which was evidenced by the occurrence of teeth chattering, writhing, jumping and wet dog shaking. As indicated in Figure 1a, the metabotropic receptor antagonists (*S*)-4C-PG and L-AP3 significantly decreased the occurrence of timed abstinence symptoms (teeth chattering and writhing). The effect for (*S*)-4C-PG appeared to be dose dependent, with the highest dose, 40 nmol day^{-1}, producing the greatest reduction in teeth chattering and writhing. L-AP3 was most effective at 8 nmol day^{-1}. Figure 1b shows the amount of time spent teeth chattering and writhing for rats treated with MK-801 and GYKI 52466. The NMDA receptor antagonist MK-801 significantly decreased the time spent in withdrawal at all doses used. The AMPA/kainate receptor antagonist GYKI 52466 did not affect the amount of time spent in withdrawal at any of the doses used.

Figure 2 illustrates the frequency of the counted abstinence symptoms, jumps and wet dog shakes. Although MK-801 tended to *increase* the number of jumps and wet dog shakes at the high dose (40 nmol day^{-1}), none of the i.c.v. EAA antagonist treatments *significantly* affected the number of jumps and wet dog shakes.

Figure 3 shows the percentage of time spent in each of the timed behaviours during the 10 min prior to naloxone administration and during the 10 min following naloxone for rats in each i.c.v. treatment group either with or without concurrent morphine treatment. As can be seen in Figure 3a and b, prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being more grooming in L-AP3 treated rats than in saline treated rats. In addition, saline-treated rats that were also given morphine reared more than rats given i.c.v. saline alone. Although activity levels (ambulating and rearing) were lower and resting [illegible] generally higher after the injection of naloxone, rats given i.c.v. EAA antagonists without morphine still behaved very similarly to rats given i.c.v. saline without morphine. The only differences observed were an increase in grooming [illegible] L-AP3-treated rats and increased activity in GYKI 52466

Discussion

The present [illegible] rats with v[illegible] leads to a [illegible] drawal. The metabotropi[illegible] were all eff[illegible] exhibiting t[illegible] writhing, v[illegible] GYKI 5246[illegible] kainate rec[illegible] withdrawal [illegible] significantly [illegible] jumping an[illegible] withdrawal [illegible] primarily b[illegible]

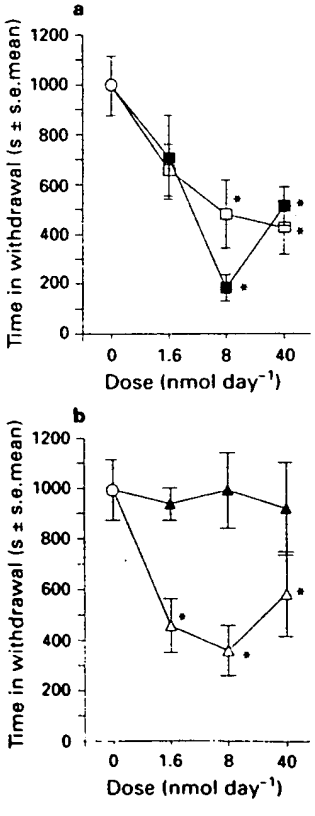
rats, as evidenced by increased ambulating, rearing grooming and decreased resting. As expected, rats dent on morphine showed significantly more naloxone-tated withdrawal, with a resultant decrease in non-awal behaviours, than rats given i.c.v. treatments In morphine-dependent rats, time in withdrawal was cantly less in L-AP3-, (*S*)-4C-PG- and MK-801-treated which coincided with an increase in ambulation in C-PG- and MK-801-treated rats.

Discussion

present results demonstrate that concurrent treatment of with various EAA antagonists and chronic morphine to a decrease in various symptoms of morphine with-wal. The NMDA receptor antagonist MK-801 and the botropic receptor antagonists (*S*)-4C-PG and L-AP3 all effective at decreasing the amount of time spent hibiting the withdrawal symptoms of teeth chattering and hing, while the AMPA/kainate receptor antagonist GYKI 52466 had no effect. Although all but the AMPA/ kainate receptor antagonist significantly reduced the timed drawal symptoms, none of the EAA antagonists cantly affected the counted withdrawal symptoms (i.e. ping and wet dog shakes). It has been suggested that drawal symptoms such as jumping are mediated arily by structures around the fourth ventricle, as evidenced by focal brain micro-injections of naltrexone and levallorphan in morphine-dependent rats (Laschka *et al.*, 1976; Koob *et al.*, 1992). In the present study, EAA antagonists were infused in very small volumes into the lateral ventricle, thus it is possible that the counted symptoms were not affected because the drugs were unable to diffuse to the appropriate brain structures around the fourth ventricle.

There was very little effect of i.c.v. EAA antagonist treatment on non-withdrawal behaviours, except for a general increase in grooming in rats given L-AP3 and increased activity in GYKI 52466-treated rats given naloxone. In general, rats given i.c.v. treatment alone rested more during the second 10 min (i.e. after naloxone), with concurrent decreases in ambulation and rearing. This is probably *not* an effect of naloxone, but rather because they had adequately explored the test box and were comfortable in the environment (a phenomenon common in untreated rats). Rats treated chronically with morphine exhibited withdrawal following the injection of naloxone, which caused a subsequent decrease in other timed behaviours. L-AP3, (*S*)-4C-PG and MK-801 all decreased withdrawal compared with saline in morphine-treated rats, with a resultant increase in ambulation in rats given MK-801 + morphine and (*S*)-4C-PG + morphine. The decrease in withdrawal allows for more time to be spent in other behaviours.

Although we have no explanation for why L-AP3 would increase grooming, it appears to be a robust effect since it occurred in every condition except in rats going through withdrawal (i.e. morphine + naloxone). The increase in activity produced by GYKI 52466 appears to be less robust since it occurred only in the group also treated with naloxone. Nonetheless, it is not expected that these behavioural effects of L-AP3 or GYKI 52466 alter our conclusions about the effects of EAA antagonists on withdrawal behaviour. GYKI 52466 did not significantly affect withdrawal, and the effects of the metabotropic receptor antagonist L-AP3 were confirmed by another metabotropic antagonist (*S*)-4C-PG, which did not significantly influence non-withdrawal behaviours. Therefore, we propose that EAA antagonist treatment affects the development of dependence directly, and not indirectly by interfering with the measurement of the withdrawal behaviours.



Mean time spent exhibiting withdrawal (teeth chattering hing) during the 40 min withdrawal period in rats given .c. morphine and i.c.v. treatment with the metabotropic antagonists (*S*)-4C-PG (□) ($n = 5-10$ per dose) and L-AP3 4–10 per dose) (a), the NMDA receptor antagonist MK- ($n = 4-10$ per dose) (b), and the AMPA/kainate receptor GYKI 52466 (▲) ($n = 4-10$ per dose) (b). ANOVA significant effects of (*S*)-4C-PG [$F_{(3,22)} = 5.124$, $P < 0.001$], $_{(3,22)} = 12.107$, $P < 0.001$] and MK-801 [$F_{(3,21)} = 6.222$, $P < 0.01$], but not GYKI 52466 [$F_{(3,20)} = 0.071$, $P > 0.05$]. differences from the control group are indicated by ($P < 0.05$, LSD *t*-test).

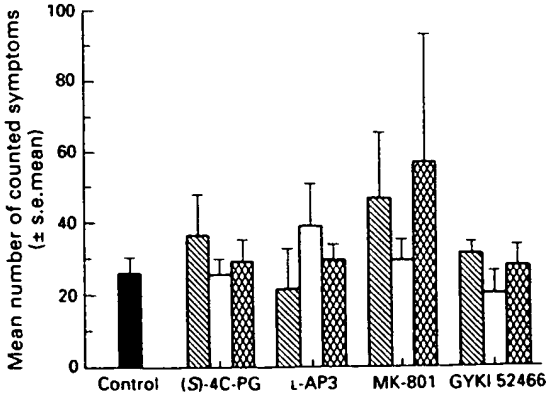


Figure 2 Frequency of counted symptoms (jumps and wet dog shakes) during the 40 min withdrawal period for rats given chronic s.c. morphine and i.c.v. treatment with the metabotropic receptor antagonists (*S*)-4C-PG ($n = 5-10$ per dose) or L-AP3 ($n = 4-10$ per dose), the NMDA receptor antagonist MK-801 ($n = 4-10$ per dose) or the AMPA/kainate receptor antagonist GYKI 52466 ($n = 4-10$ per dose). Kruskal-Wallis ANOVA for non-parametric data revealed no significant effects of any of the EAA antagonists. (*S*)-4C-PG, $H_{(3,22)} = 0.803$, $P > 0.05$; MK-801, $H_{(3,21)} = 0.747$, $P > 0.05$; GYKI 52466, $H_{(3,20)} = 2.580$, $P > 0.05$; L-AP3, $H_{(3,23)} = 2.142$, $P > 0.05$. Solid columns, 0 nmol day^{-1}; diagonal hatched columns, 1.6 nmol day^{-1}; open columns, 8 nmol day^{-1}; cross-hatched columns, 40 nmol day^{-1}.

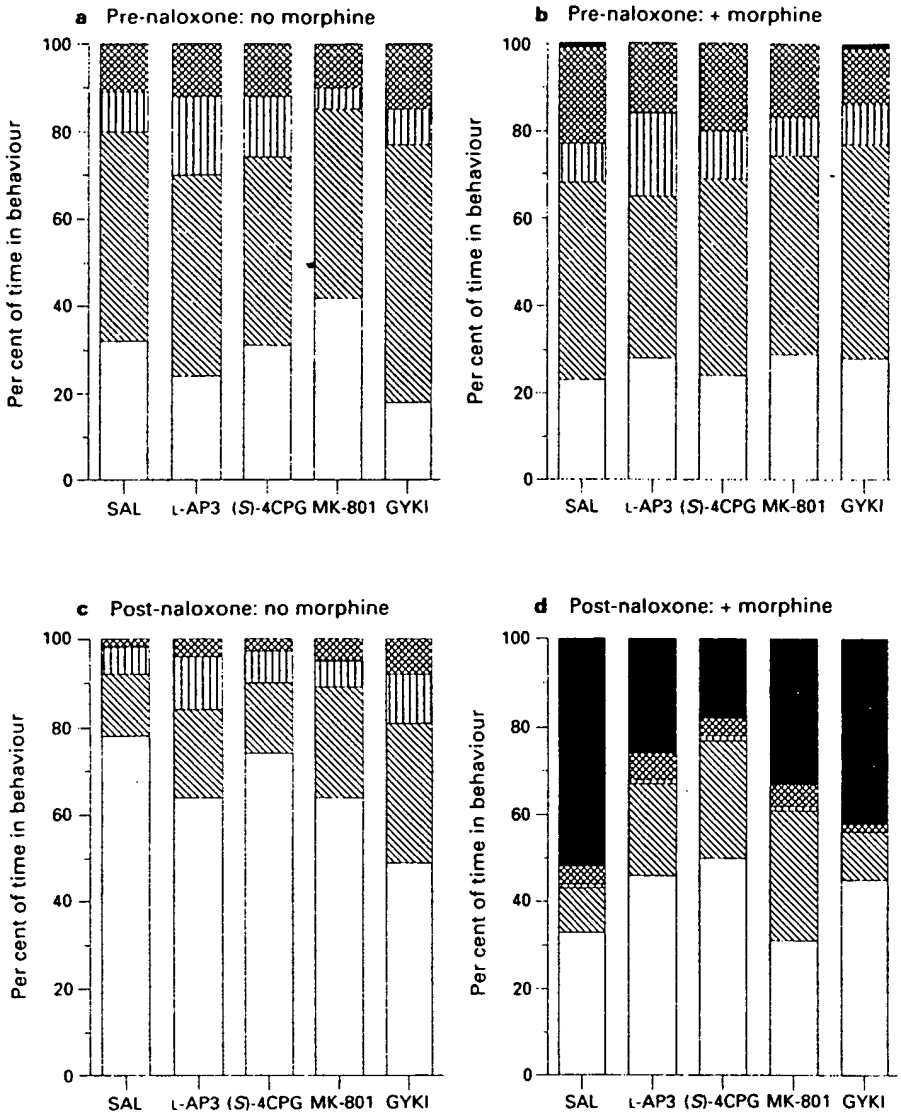


Figure 3 Percentage of time in non-withdrawal and withdrawal behaviours in rats treated chronically with either saline or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 (EAA antagonists) i.c.v. alone (i.e. no s.c. morphine; $n = 6-8$ per i.c.v. treatment) (a) or with s.c. morphine ($n = 4-10$ per i.c.v. treatment) (b) during the 10 min *prior* to the injection of naloxone; and in rats given chronic i.c.v. saline or EAA antagonists alone (c) or with s.c. morphine (d) during the 10 min *after* the injection of naloxone. Prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being increased grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (a and b). With the addition of morphine, saline-treated rats exhibited more rearing than rats not treated with morphine (planned comparison$_{(1,52)}$, $P<0.05$) (b). After the injection of naloxone, rats not dependent on morphine generally rested more than in the first 10 min. Non-dependent rats in all i.c.v. treatment groups behaved similarly, with the only differences being more grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (c) and more activity in GYKI 52466-treated rats as evidenced by significantly more ambulating (diagonal hatched columns), rearing (cross-hatched columns) and grooming (vertically hatched columns) and significantly less resting (open columns) (planned comparison$_{(1,52)}$, $P<0.05$) (c). Morphine-dependent rats showed a significant increase in withdrawal behaviours (solid columns) regardless of i.c.v. treatment after the injection of naloxone (planned comparison$_{(1,52)}$, $P<0.05$) (d). However, L-AP3, (*S*)-4C-PG and MK-801 all significantly decreased the percentage of time spent in withdrawal behviours, with a concurrent increase in ambulation in (*S*)-4C-PG- and MK-801-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (d).

Previously, it has been observed that concurrent treatment of rats with the non-selective EAA antagonist kynurenic acid (Marek *et al.*, 1991a) or the non-competitive NMDA antagonist MK-801 (Marek *et al.*, 1991b; Trujillo & Akil, 1991) with daily injections of morphine attenuated the development of tolerance to morphine's analgesic effects. MK-801 also alleviated the severity of some symptoms of the precipitated withdrawal syndrome (Trujillo & Akil, 1991). Furthermore, some investigators have found that acute treatment with kynurenic acid and MK-801 only on the day of testing (i.e. not concurrently with morphine) is effective in decreasing the severity of some withdrawal symptoms (Rassmussen *et al.*, 1991a,b; Tanganelli *et al.*, 1991), while other did not find this acute administration effective (Trujillo & Akil, 1991). While each of these above studies assessed the effects of *systemically* administered EAA antagonists, in additional experiments (unpublished data), we have found that acute *i.c.v.* injections of EAA antagonists on day 7 prior precipitation of withdrawal failed to attenuate severity abstinence symptoms, lending support to the latter group.

Hyperactivity in the locus coeruleus (LC) has been shown to be correlated with the morphine withdrawal syndrome (Aghajanian, 1978; Valentino & Wehby, 1989). Central administration of non-specific, NMDA-selective and AMPA

-selective EAA antagonists into either the lateral ventricle or the locus coeruleus has been found to decrease the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Akaoka & Aston-Jones, 1991), with the effects produced by the non-specific EAA antagonist. Conversely, systemic administration of selective NMDA receptor antagonists was unable to affect the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Rasmussen *et al.*, 1991a).

The present data indicate that chronic i.c.v. administration of the selective metabotropic EAA receptor antagonists (*S*)-4C-PG and L-AP3 was at least as effective in attenuating the severity of the morphine withdrawal syndrome as antagonists selective for the NMDA receptors. It is noteworthy that the most effective treatment was 8 nmol day^{-1} L-AP3. This strong effect of L-AP3 may have resulted from a possible additive effect of L-AP3 at both metabotropic and NMDA receptors. There is evidence that L-AP3 may have non-selective effects at the NMDA receptor as well as its proposed major action at the metabotropic receptor (Birse *et al.*, 1993). The significant dose-dependent effects of the highly selective metabotropic receptor antagonist (*S*)-4C-PG, however, do suggest an important role of metabotropic glutamate receptors in the development of morphine dependence. The failure of GYKI 52466 to influence precipitated withdrawal suggests that AMPA/kainate receptors do not play an important role in morphine dependence. Although it is possible that GYKI 52466's ineffectiveness could be explained by rapid metabolism *in vivo*, this is unlikely because the drug was chronically infused. Furthermore, it has been demonstrated, using an osmotic pump infusion method similar to that used in the present study, that GYKI 52466 is as effective at attenuating excitatory amino acid induced seizures on the 14th day of infusion as it is on the third day of infusion (Steppuhn & Turski, 1993).

Activity at specific metabotropic receptor subtypes stimulates phosphatidylinositol (PI) hydrolysis and leads to the production of the intracellular messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Chronic opioid use may alter production of these intracellular messengers and thus elicit long-term changes which contribute to opioid tolerance and dependence. There is evidence that acute morphine treatment stimulates PI hydrolysis (Raffa & Martinez, 1992), while chronic morphine treatment inhibits PI hydrolysis (Dixon *et al.*, 1990). (*S*)-4C-PG and L-AP3 antagonism of the metabotropic glutamate receptor during morphine treatment may prevent cellular changes associated with persistent phosphatidylinositol hydrolysis, and consequently reduce withdrawal symptoms that are associated with these cellular changes. Other metabotropic receptor subtypes inhibit production of cAMP (Schoepp & Conn, 1993). It is well established that both acute and chronic opioid use also affect the production of cAMP (Collier, 1980, 1983; Sharma *et al.*, 1975). Thus it is possible that (*S*)-4C-PG and L-AP3 antagonism of metabotropic receptors prevented changes in the cAMP system associated with chronic opioid use. Currently, we are determining whether changes in the PI system or changes in the cAMP system associated with activation of metabotropic receptors are important for the development of tolerance and dependence with chronic opioid use.

Thus, the present study indicates that both NMDA and metabotropic glutamate receptors may be involved in the development of dependence with chronic morphine use. Both NMDA and metabotropic glutamate receptors are associated with changes in intracellular second messenger systems. It is therefore hypothesized that NMDA and metabotropic glutamate antagonists are effective because they prevent changes in second-messenger systems associated with chronic opioid use.

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Excitatory amino acid antagonists and their potential for the treatment of ischaemic brain damage in man

Exhibit W (10/644, 645)

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1 A wide range of therapeutic strategies has been explored in humans and experimental animals with the aim of improving outcome after brain ischaemia but few have shown convincing clinical benefit.

2 The massive increase in the extracellular concentration of glutamate which occurs in cerebral ischaemia is a key component in the sequence of neurochemical events which leads to neuronal death. Pharmacological blockade of the action of glutamate at the *N*-methyl-D-aspartate (NMDA) receptor, (the glutamate receptor subtype principally involved in the neurotoxic effects of the amino acid) provides a novel therapeutic approach to cerebral ischaemia.

3 The effects of NMDA receptor antagonists in animal models of focal cerebral ischaemia are uniquely consistent, *viz*, a marked reduction in the amount of irreversible ischaemic damage irrespective of the species, the model of cerebral ischaemia, when the animals are sacrificed after the ischaemic episode, whether ischaemia is permanent or temporary and followed by reperfusion and which particular NMDA antagonist was employed.

4 NMDA receptor antagonists have marked effects on brain function in normal animals. The balance between these potential adverse effects and the anti-ischaemic efficacy of these drugs will ultimately determine the clinical utility of this class of drugs.

5 The data which are reviewed provide the basis for the current clinical evaluation of NMDA receptor antagonists in stroke and head trauma.

Keywords *N*-methyl-D-aspartate ischaemic brain damage glutamate receptor

Introduction

The importance of cerebral ischaemia is a reflection of the frequency of cerebrovascular disease in advanced societies and the severity of its sequelae. Cerebrovascular disease ranks third (after cancer and heart disease) as the cause of death in Western Europe and North America and is the major cause of handicap in the adult population. Approximately 500,000 people in the U.K. are presently incapacitated by the neurological effects of cerebral ischaemia.

Focal cerebral ischaemic damage (stroke) results from a reduction in cerebral blood flow to a discrete brain area. The origin of the ischaemic episode may be occlusive (due to *in situ* arterial thrombosis), embolic or haemorrhagic. In some patients it is due to a combination of proximal vascular narrowing and impairment of total cerebral blood flow, e.g. due to a sudden reduction in cardiac output. Ischaemic brain damage is a feature of a number of clinical conditions other than stroke, most notably head injury, prolonged seizures, cardiac arrest, perinatal hypoxia, etc. These conditions provide additional patients who may benefit from excitatory amino acid receptor antagonists and, as in the case of head injury, clinical populations in which the efficacy and potential adverse reactions of these class of drugs may be readily studied.

Stroke therapy can be directed at a wide range of pathophysiological mechanisms and there has long been particular interest in medical and surgical therapies designed to improve cerebral blood flow to the ischaemic tissue. Drugs which putatively increase flow to ischaemic tissue, such as nimodipine, are of clear benefit to subarachnoid haemorrhage patients who are at high risk of

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delayed ischaemia due to vasospasm and reduced cerebral blood flow (Pickard *et al.*, 1989). Nimodipine may also be of benefit in stroke, but the evidence is more controversial (Gelmers *et al.*, 1988). In addition, there has long been concern that simply increasing blood flow to ischaemic brain tissue may have adverse consequences such as increased cerebral oedema, haemorrhagic transformation or generation of free radicals (Hossmann, 1982).

The role of excitatory amino acids in the genesis of ischaemic neuronal injury

The concept that blockade of excitatory amino acid receptors attenuates the transmembrane ionic fluxes that lead to neuronal death provides a therapeutic strategy that does not depend upon improvement in cerebral blood flow. High concentrations of glutamate are neurotoxic (Choi, 1991; Lucas & Newhouse, 1957; Rothman & Olney, 1986). From extensive investigations in cell cultures (for review see Choi, 1991), the neurotoxic effects of glutamate appear to be mediated predominantly via activation of the *N*-methyl-D-aspartate (NMDA) receptor subtype although the contribution of non-NMDA receptors is becoming increasingly recognised (see Choi, 1991; Choi *et al.*, 1988; Frandsen *et al.*, 1989; Michaels & Rothman, 1990). Recent evidence suggests that the generation of nitric oxide via NMDA receptor activation may contribute to neuronal damage (Dawson *et al.*, 1991).

In experimental cerebral ischaemia, there is a marked, immediate increase in the extracellular concentrations of glutamate and aspartate, irrespective of the nature and primary cause of the ischaemic episode (Figure 1). Ischaemia induced elevations in excitatory amino acids occur in all brain areas which have been investigated and in response to all experimental approaches employed to provide low levels of cerebral blood flow (i.e. global ischaemia, middle cerebral artery occlusion, CNS trauma, subdural haemorrhage) (McCulloch *et al.*, 1991). The elevation in extracellular glutamate in ischaemia is due to an increased release from neurones, to an impaired uptake of glutamate into neurons and astrocytes in the ischaemic tissue and to reversal of the uptake mechanism (Nicholls & Attwell, 1990). The relationship between extracellular glutamate and cerebral blood flow is a threshold type relationship with elevation in glutamate being triggered by blood flow reduction below 20 ml 100 g^{-1} min^{-1} (Shimada *et al.*, 1989), suggesting that glutamate threatens cerebral tissue in the ischaemic penumbra as well as in the ischaemic core. The blood flow threshold for irreversible damage to neurones is time dependent. Cerebral blood flow of 17 ml of blood 100 g^{-1} of brain tissue min^{-1} (or 35% of basal levels of cerebral blood flow) must be sustained for 3 h or more to produce damage, whereas neuronal damage occurs if there is a complete cessation of cerebral blood flow beyond a few minutes (Jones *et al.*, 1981).

The actions of excitatory amino acids such as aspartate and glutamate are mediated by at least four distinct receptor subtypes. NMDA, kainate and 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors have been defined in terms of their selective affinity for the appropriate agonists and these glutamate receptor subtypes are all associated with receptor operated ion channels. A fourth glutamate receptor subtype ('the metabotropic receptor') has been identified recently and is linked to phosphoinositide metabolism (Lodge & Collingridge, 1990).

There are a number of distinct sites within the NMDA receptor ion channel complex at which drugs may act to attenuate the effects of glutamate (Figure 2) (see Foster & Fagg, 1987). Conceptually, the most simple site at which NMDA antagonists can exert their action is the neurotransmitter recognition site for glutamate and NMDA, the most potent of these competitive NMDA

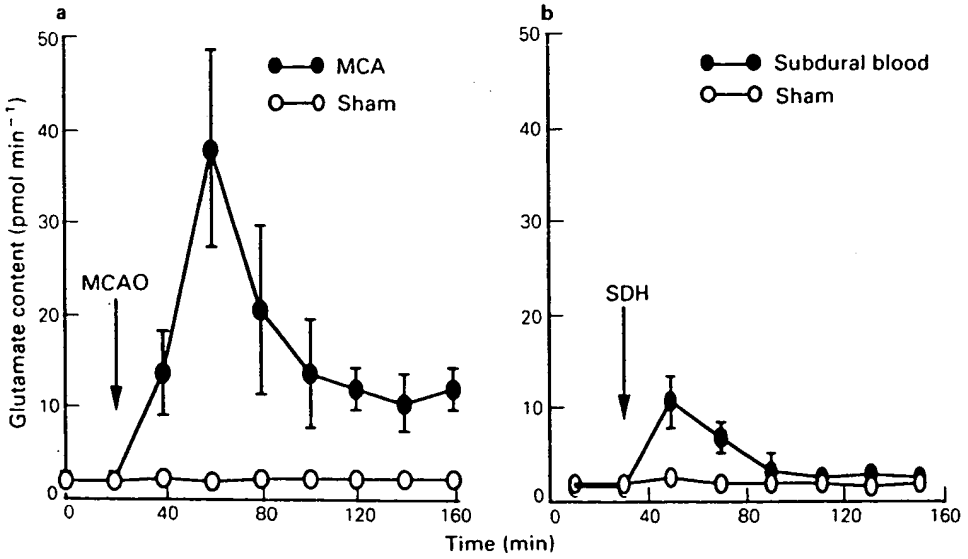

Figure 1 Extracellular glutamate concentrations are elevated in focal cerebral ischaemia produced by middle cerebral artery occlusion (a) and after induced subdural haematoma (b). Data are from microdialysis probes in the rat cerebral cortex. Dialysates were collected in 20 min fractions (2.5 μl min^{-1}). After middle cerebral artery occlusion, there is approximately a 20-fold increase at peak in extracellular glutamate concentrations; after subdural haematoma, there is a five-fold increase. Redrawn from the data of Butcher *et al.* (1990) and Bullock *et al.* (1991).

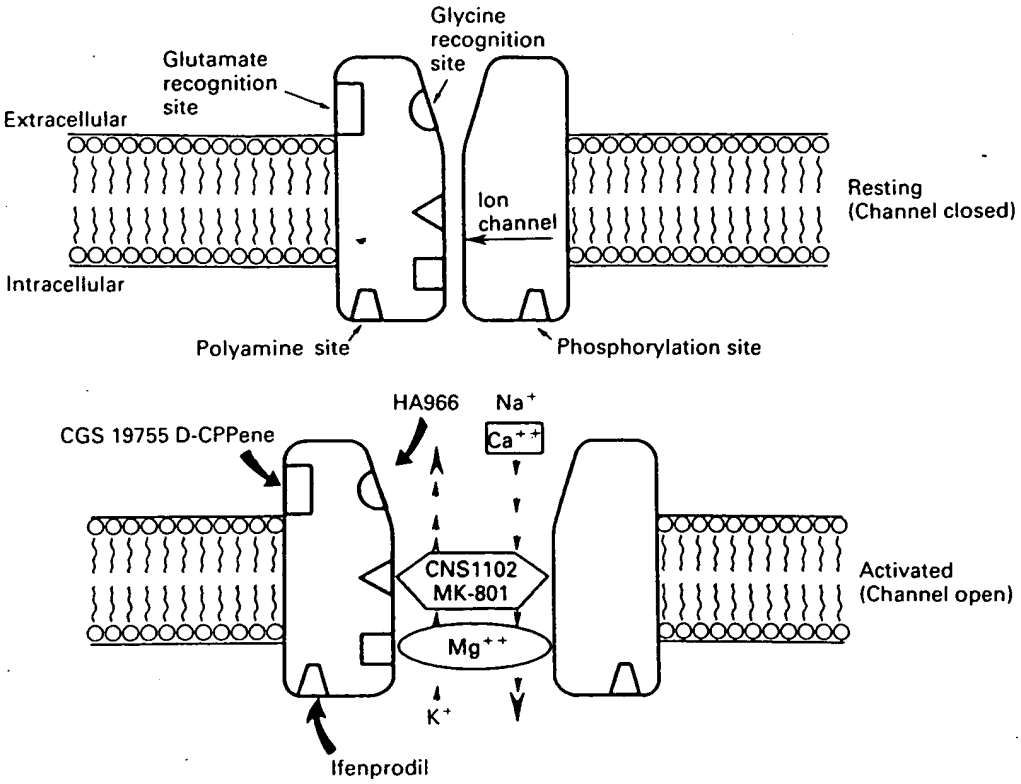

Figure 2 Diagrammatic representation of the NMDA receptor complex. Blockade of the NMDA receptor can be achieved at multiple, pharmacologically distinct sites. Competitive NMDA antagonists (e.g. D-CPPene, CGS 19755) act at the agonist recognition site. Non-competitive NMDA antagonists (e.g. MK-801, CNS 1102) and Mg^{++} act at distinct sites within the ion channel. Blockade of the actions of NMDA can also be achieved via blockade of the glycine recognition site (e.g. with HA-966) or polyamine site (e.g. with ifenprodil). Redrawn from Foster & Fagg (1987).

antagonists which have been studied in models of ischaemia being *cis*-4-phosphonomethyl-2-piperidine-carboxylic acid (CGS 19755) and D-3(2-carboxypiperazin-4-yl) propenyl-1-phosphonic acid (D-CPPene) (Aebischer *et al.*, 1989; Lehmann *et al.*, 1988). Agents such as MK-801, CNS 1102 and phencyclidine (PCP) interact with a site within the ion channel of the NMDA receptor to produce a non-competitive blockade of the actions of glutamate (Kemp *et al.*, 1987). Agents such as 7-chloro-kynurenic acid and 3-amino-1-hydroxy-2-pyrrolidone [(+)HA 966] appear to attenuate the effects of NMDA receptor agonists by acting at a site through which glycine allosterically enhances NMDA receptor function (Kemp *et al.*, 1988; Singh *et al.*, 1990). Other allosteric sites within the NMDA receptor (the 'polyamine site') may be involved in the action of ifenprodil and related compounds to the NMDA receptor complex (Carter *et al.*, 1989). The opening of the NMDA receptor-ion channel is voltage-dependent by virtue of blockade with physiological concentrations of magnesium; membrane depolarisation at the onset of cerebral ischaemia relieves the magnesium block of the NMDA ion channel.

The existence of multiple, pharmacologically active sites within the NMDA receptor ion channel complex is not of esoteric neuropharmacological interest. The different sites within the NMDA receptor complex at which non-competitive antagonists (such as MK-801) and competitive antagonists (such as D-CPPene) act, and the influence of glutamate upon their interactions with their specific binding sites may have a crucial bearing on the efficacy of these two types of NMDA antagonists in cerebral ischaemia and their potential for adverse effects on CNS function. Non-competitive antagonists such as MK-801 produce a use-dependent blockade, in which the binding of the drug to its recognition site in the ion channel and the resulting NMDA blockade are markedly enhanced by high concentrations of glutamate (Kemp *et al.*, 1987; Wong *et al.*, 1986). In contrast, the NMDA receptor blockade produced by competitive antagonists such as DCPPene can be overcome or reduced by increasing concentrations of glutamate (Kemp *et al.*, 1987). In cerebral ischaemia the presence of high extracellular glutamate levels should intensify the blockade produced by non-competitive NMDA antagonists such as MK-801, but could potentially counteract the blockade produced by competitive antagonists such as D-CPPene

Anti-ischaemic efficacy of NMDA receptor antagonists in experimental animals

Focal cerebral ischaemia

The effects of NMDA receptor antagonists in experimental models of focal cerebral ischaemia can be readily summarised, *viz.* these drugs effect a marked reduction in the amount of irreversible ischaemic damage irrespective of the species, the model of cerebral ischaemia when the animals are sacrificed after the ischaemic episode, whether ischaemia is permanent or temporary and followed by reperfusion, and irrespective of the particular site within the NMDA receptor at which the drug acts.

The consistency of view which has emerged from the

use of NMDA antagonists in experimental focal ischaemia is unique for any pharmacological class of anti-ischaemic drug. The anti-ischaemic efficacy of NMDA antagonists in experimental focal ischaemia (as distinct from global ischaemia, *vide infra*) is not due to the focality of the ischaemic insult but to its moderate severity as distinct from the complete (or near complete) absence of blood flow to the brain in most reliable global models. In middle cerebral artery occlusion models of focal ischaemia, the failure of NMDA antagonists to protect the basal ganglia has been attributed to the much lower levels of blood flow which occur after occlusion of the middle cerebral artery in the caudate nucleus relative to the cerebral cortex. The lack of protection in the caudate nucleus indicates that a minimal level of cerebral blood flow is required for anti-ischaemic efficacy of NMDA antagonists (McCulloch *et al.*, 1991).

Cat The clearest evidence of the potency of NMDA antagonists as anti-ischaemic agents has emerged from studies of their effects in permanent middle cerebral artery (MCA) occlusion in the cat where the volume of ischaemic damage has been comprehensively assessed (Figure 3). Pretreatment with a competitive antagonist (D-CPPene) or a non-competitive antagonist (MK-801) or polyamine site blockers (ifenprodil and *d*-(4-chlorophenyl)-4-[(4-fluorophenyl)methyl]-1-piperidine ethanol (SL 82.0715)), administered within 5 min of the occlusion, markedly reduces the volume or irreversible ischaemic brain damage in the cerebral hemisphere (Figure 4) (Bullock *et al.*, 1990; Chen *et al.*, 1991; Gotti *et al.*, 1988; Ozyurt *et al.*, Uematsu *et al.*, 1991).

A critical issue for all potential anti-ischaemic compounds is that of how long after the onset of the ischaemic episode these agents are able to prevent ischaemic damage occurring. It is self-evident that for a drug to be effective it must be present in the ischaemic tissue in adequate concentration during the time window of the therapeutic

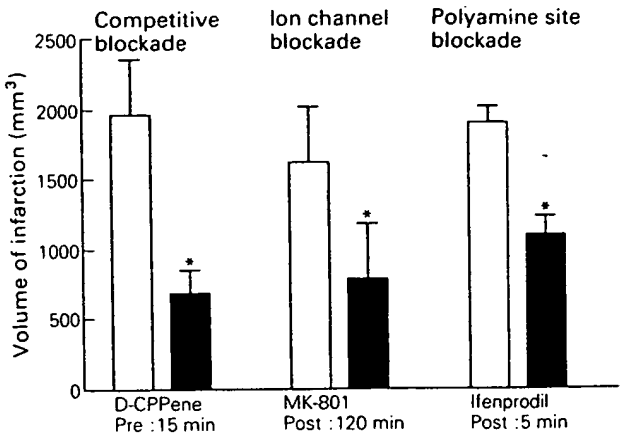

Figure 4 NMDA receptor antagonists markedly reduce the volume of ischaemic brain damage which results from permanent middle cerebral artery (MCA) occlusion. The magnitude of neuroprotection is similar with competitive blockade (D-CPPene, 15 mg kg^{-1}, i.v. 15 min before MCA occlusion), ion channel blockade (MK-801 5 mg kg^{-1}, i.v. 120 min after MCA occlusion), and polyamine site blockade (ifenprodil, 16.7 μg kg^{-1} min^{-1}, i.v. initiated 5 min after MCA occlusion). Data are presented as mean ± s.e. mean (n = 6–13 per group). □ vehicle, ■ drug. Original data are from Chen *et al.* (1991), Gotti *et al.* (1988) and Park *et al.* (1988). Reproduced from McCulloch & Iversen (1991) with permission.

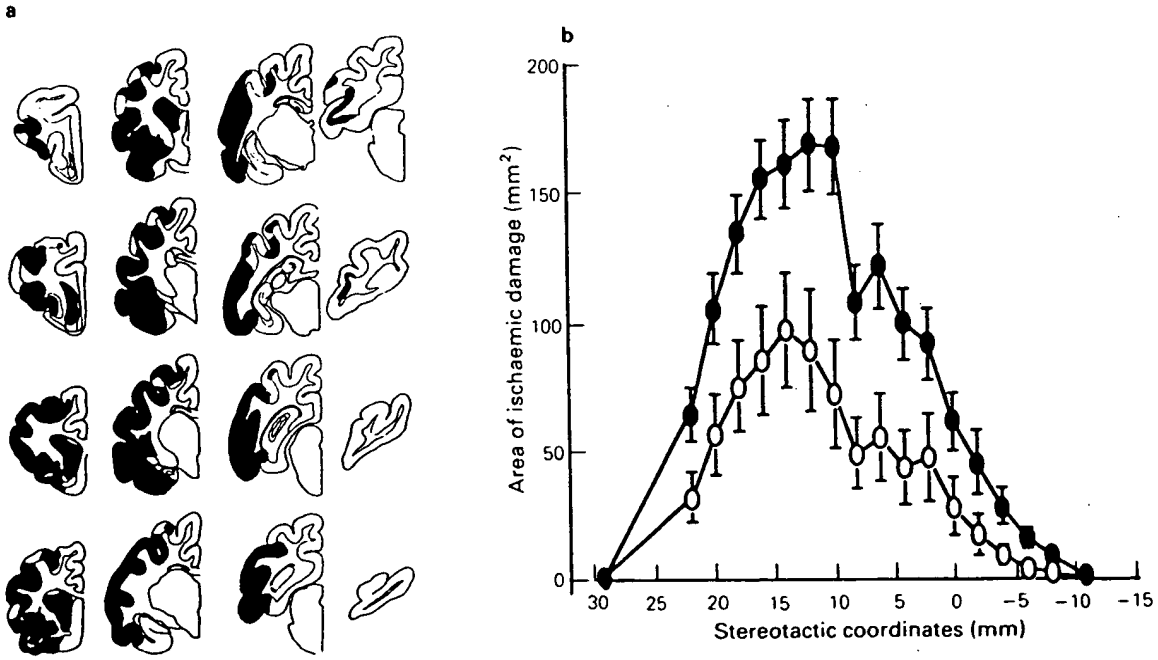

Figure 3 Effect of MK-801 upon ischaemic brain damage after middle cerebral artery occlusion in the cat: volumetric assessment of ischaemic brain damage. a) The areas of ischaemic brain damage (solid black) assessed with light microscopy, are charted onto line drawings for 16 predetermined coronal planes. b) Effect of MK-801 (5 mg kg^{-1} 30 min prior to MCA occlusion) on the area of ischaemic damage in the 16 coronal planes. There are significant differences between vehicle (•) and MK-801 (○) treatment at each coronal plane. Data are mean ± s.e. mean (n = 9 in each group). The volumes of ischaemic damage calculated from the areas and the known stereotactic co-ordinates were vehicle 3231 ± 394 mm^3 and MK-801 1602 ± 445 mm^3 ($P < 0.01$). Original data from Ozyurt *et al.* (1988) reproduced from McCulloch *et al.* (1991) with permission.

opportunity (i.e. less than 3 h in the cat MCA occlusion model (even with penumbra level of blood flow). The chemistry of the drug has considerable bearing on what extent (and how quickly) plasma drug levels are reflected in ischaemic cerebral tissue. For a highly lipophilic agent such as MK-801 with rapid CNS entry, the low levels of blood flow in ischaemic tissue only slightly delay its appearance in ischaemic tissue (e.g. 5 min after administration, the level in ischaemic tissue is 50% of that in the cerebellum) (Wallace *et al.*, 1992). By virtue of its rapid brain uptake, MK-801 first administered 2 h after the onset of ischaemia is as effective as pretreatment in reducing the volume of ischaemic brain damage in the cat MCA occlusion model (Park *et al.*, 1988b). In contrast for hydrophilic molecules such as D-CPPene (and almost all other competitive NMDA antagonists presently available) the rate at which equilibrium is achieved between plasma and CNS is extremely slow (half-time of CNS uptake of 60 min or more). The slow diffusion across the blood-brain barrier probably accounts for the lack of a significant effect of D-CPPene when treatment is initiated 1 h after MCA occlusion (Chen *et al.*, 1991).

There is evidence which suggests that the magnitude of neuroprotection offered by MK-801 is broadly similar in temporary MCA occlusion in the cat (2 h occlusion followed by 4 h reperfusion) (Uematsu *et al.*, 1991) to that observed with 6 h of permanent MCA occlusion (Ozyurt *et al.*, 1988; Park *et al.*, 1988a). Furthermore, nimodipine treatment together with MK-801 appears to result in greater reductions in the amount of brain damage than does MK-801 alone in the cat focal ischaemia – reperfusion model (Uematsu *et al.*, 1991).

Primate In the single study available at present, post-ischaemic treatment with MK-801 reduces the amount of brain damage and improves neurological outcome after temporary focal ischaemia in non-human primates (Zabramski *et al.*, 1991).

Rabbit The investigations of the efficacy of NMDA antagonists in focal cerebral ischaemia in rabbits, though numerically limited, contain a number of interesting features. They provide one of the few reliable demonstrations of the efficacy of MK-801 in a model of embolic stroke (Kochhar *et al.*, 1988). Functional recovery after MK-801 treatment in spinal cord ischaemia was first shown in the rabbit (Kochhar *et al.*, 1988). Dextromethorphan and its active metabolite, dextrorphan, which are weak, non-competitive NMDA antagonists, have been most extensively examined in a rabbit model of temporary focal ischaemia followed by reperfusion. Both these agents, with pretreatment and with treatment initiated at the start of reperfusion after 1 h of ischaemia, reduce the amount of ischaemic damage (assessed with histology), the amount of oedema (assessed with MRI) and improve functional recovery (assessed with somatosensory evoked responses) (George *et al.*, 1988; Steinberg *et al.*, 1988, 1991). It should be emphasised that the threshold anti-ischaemic dose of these agents in the rabbit is 15 mg kg^{-1} (i.v.) in the first hour of treatment (Steinberg *et al.*, 1991), compared with the antitussive dose in man of 0.2–0.4 mg kg^{-1} by mouth (3–4 times daily).

Rat The efficacy of NMDA antagonists in rat model of focal cerebral ischaemia has been confirmed in numerous reports. There is overwhelming evidence that non-competitive NMDA antagonists (MK-801, TCP, PCP) reduce the amount of ischaemic damage after MCA occlusion in the rat (Bielenberg & Beck, 1991; Dirnagl *et al.*, 1990; Gill *et al.*, 1991; Gotti *et al.*, 1988; Park *et al.*, 1988a; Roussel *et al.*, 1992). There is growing evidence for the view that competitive NMDA antagonists, glycine antagonists, polyamine site antagonists and the systemic administration of Mg^{++} are also effective in focal ischaemia in the rat (Gill *et al.*, 1991; Gotti *et al.*, 1988; Izumi *et al.*, 1991; Park *et al.*, 1991; Simon & Shiraishi, 1990). The volume of tissue which can be salvaged from irreversible ischaemic damage with NMDA antagonists is approximately 50% of the infarction volume in untreated rats; the maximum anti-ischaemic effects of the drugs are broadly similar irrespective of their precise site of action within the NMDA receptor complex. Marked neuroprotection with MK-801 and PCP is observed despite the marked hypotension which it produces in halothane-anaesthetised rats (Bielenberg & Beck, 1991; Park *et al.*, 1988a). Hypotension would tend to exacerbate damage by reducing blood flow in the ischaemic penumbra to even lower levels (Osborne *et al.* 1987). Drug-induced hypotension is the probable cause of the U-shaped dose-response curve noted with PCP and MK-801 in the rat MCA occlusion model (Bielenberg & Beck, 1991; Gill *et al.*, 1991). There is evidence (see Dirnagl *et al.*, 1990; Roussel *et al.*, 1990, 1992) that the magnitude of response to MK-801 and kynurenate may be somewhat smaller in spontaneously hypertensive animals probably because the ischaemic insult after MCA occlusion is more severe in the hypertensive strain than in normotensive animals (Roussel *et al.*, 1992).

A recent report indicates that blockade of glutamate receptors other than the NMDA subtype with NBQX can also reduce ischaemic damage in the rat (Gill *et al.* 1992).

Perinatal hypoxia

Perinatal hypoxia, like focal cerebral ischaemia, is another area where there is convincing evidence of reductions in ischaemic brain damage. MK-801, kynurenate and dextromethorphan all putatively reduce the amount of damage produced by hypoxia and unilateral carotid artery occlusion in neonatal rats (Andiné *et al.*, 1988; Hattori *et al.*, 1989; McDonald *et al.*, 1987; Olney *et al.*, 1989; Prince & Feesser, 1988). MK-801 treatment is of benefit even when initiated up to 75 min after the hypoxic episode (Hattori *et al.*, 1989; McDonald *et al.*, 1989). Despite their undoubted efficacy in neonatal models of hypoxia, the medico-legal problems associated with administration of new drugs to brain damaged infants effectively preclude the use of NMDA antagonists in this clinical area at present (particularly within litigious North America).

Global cerebral ischaemia

The pivotal investigations on anti-ischaemic efficacy of selective NMDA antagonists were that MK-801 could

protect the hippocampus of the gerbil from the effects of global ischaemia (Gill *et al.*, 1987). The present status of NMDA receptor antagonists in animal models of global ischaemia can be summarised readily. In all of the studies of global ischaemia in large animals (dogs, cats and primates) no benefit has been demonstrated. In studies of global ischaemia in rodents (rats and gerbils), while the balance of evidence, in numerical terms, favours the view that NMDA antagonists reduce delayed damage to the hippocampus, all positive reports attract criticisms that the benefit observed is indirect (i.e. due to anti-convulsant effects, drug-induced hypothermia). The severity of the ischaemia between different models of global ischaemia appears to provide the best explanation for divergent observations between different investigations (McCulloch *et al.*, 1991).

Only MK-801 has been systematically studied in large animal models of global ischaemia. In dogs, MK-801 fails to alter neurological deficits and the amount of hippocampal damage produced by 11 min global ischaemia (occlusion of ascending aorta) (Michenfelder *et al.*, 1989) or a model of prolonged (17 min) cardiac arrest with a variety of treatment paradigms (Sterz *et al.*, 1989). Similarly, in cats, MK-801 does not improve outcome (neurological deficit and neuropathology in the cortex, hippocampus and cerebellum) after 18 min cardiac arrest (Fleischer *et al.*, 1989). In a study of non-human primates with 17 min of ischaemia, MK-801 again did not provide any evidence of amelioration of the ischaemic damage to the CNS (Lanier *et al.*, 1990).

The influence of excitatory amino acid antagonists on the delayed degeneration of hippocampal CA1 pyramidal neurons in the gerbil and the rat has been the subject of intense investigation and controversy. A clear understanding of the biological and technical bases for divergent results from different laboratories is beginning to emerge. The crucial determinant of whether NMDA antagonists will be effective in ischaemia (whether focal or global) appears to be the severity of the insult and its impact on energy state (see Siesjö & Bengtsson, 1989; Wieloch *et al.*, 1989). In many models of global ischaemia and in the ischaemic core of a focal insult, complete energy failure occurs and NMDA antagonists are not efficacious. In contrast, in the ischaemic penumbra (and possibly in global models where benefit is reported with NMDA antagonists), energy state is less markedly disturbed and NMDA antagonists are clearly efficacious (see Siesjö & Bengtsson, 1989; Wieloch *et al.*, 1989). The difference between partial and complete breakdown of energy production in a diffuse insult like global ischaemia is likely to be highly marginal and extremely sensitive to a number of subtle factors such as anaesthetics, nutritional state, gender, strain (for discussion see Meldrum, 1990). Furthermore, it is now generally accepted that the small differences in brain temperature during and after transient ischaemia dramatically modify the amount of delayed neuronal damage (see Busto *et al.*, 1987; Minanisawa *et al.*, 1990). Buchan & Pulsinelli (1990) produced overwhelming evidence that the ability of MK-801 to provide neuroprotection in the gerbil was inextricably linked to hypothermia. Irrespective of how meticulously temperature is controlled (see Gill & Woodruff, 1990) there will always be concern that drug efficacy is due to hypothermia during the chronic survival period in global ischaemia models (Buchan & Pulsinelli, 1990).

Although NMDA antagonists are not effective in preventing delayed neuronal death after *severe* global ischaemia, NBQX which blocks non-NMDA glutamate receptors has recently been shown to markedly reduce damage to the hippocampus and other brain areas in these severe models (Buchan *et al.*, 1991; Nellgård & Wieloch, 1992).

NMDA receptor antagonists as clinically useful drugs

Excitatory amino acid antagonists are among a wide range of compounds presently being developed as neuroprotective agents. There is an enormous list of drugs and lead compounds at various stages of preclinical or clinical development, e.g. aminosteroids, free radical scavengers, various ion channel blockers, kappa opiate agonists, naftidrofuryl, gangliosides, 5-hydroxytryptamine$_{1A}$ ($5HT_{1A}$) agonists, $5HT_2$-receptor antagonists, α_2-adrenoceptor antagonists, cyclo-oxygenase and lipo-oxygenase inhibitors and others (Ginsberg & Scheinberg, 1991).

It is worth emphasising that among the different pharmacological classes of anti-ischaemic drugs, NMDA antagonists occupy a unique position; for no other class is there such a vast, consistent literature which documents anti-ischaemic efficacy. It is now generally accepted that NMDA blockade reduces brain damage in experimental focal ischaemia irrespective of,

(a) the species used,
(b) the experimental design (anaesthesia, chronic or acute survival, etc.)
(c) the particular site within the NMDA receptor complex at which blockade is achieved,
(d) or whether drug treatment is initiated before or in the first few hours after onset of ischaemia.

Anti-ischaemic efficacy is only one element in the selection of drugs for clinical evaluation. Safety and adverse effects are also of paramount importance in determining the utility of new drugs. It is already clear that the actions of NMDA antagonists (other than those relating to their ability to reduce brain damage) will influence the selection of the clinical target and the design of the clinical trials.

Competitive and non-competitive NMDA antagonists (MK-801 and CPP) depress respiration and induce hypercapnia (Kurumaji *et al.*, 1989). MK-801 increases blood pressure in conscious rats and chloralose-anaesthetised cats (Kurumaji *et al.*, 1989; Ozyurt *et al.*, 1988) but markedly decreases blood pressure in halothane anaesthetised rats (Bielenberg & Beck, 1991; Park *et al.*, 1988a); at high doses, D-CCPene induces hypotension in chloralose-anaesthetised cats (Bullock *et al.*, 1990b; Chen *et al.*, 1991). While these effects present minimal difficulties in some conditions (e.g. head injury patients already on a ventilator in an intensive care unit), in others (e.g. elderly stroke patients with other cardiovascular complications), they may restrict their use.

The administration of NMDA receptors antagonists alters the behaviour of all experimental animals studied hitherto including non-human primates (France *et al.*, 1989; Koek *et al.*, 1988). In primates, the behavioural

effects of NMDA antagonists include disruption of learning and memory, ataxia, sedation and ultimately anaesthesia. The central issue for clinical trials is not whether the drugs induce behavioural changes but the concentration at which the behavioural changes are manifest relative to the therapeutic doses. With non-competitive antagonists typified by MK-801, behavioural alterations are apparent at concentrations similar to those required for anti-ischaemic efficacy. For competitive antagonists, behavioural alterations occur at concentrations three to ten times greater than those required for anti-ischaemic effects and there may be a wider separation for polyamine site antagonists such as ifenprodil (compare the data for the mouse of Koek & Colpaert (1990) and Gotti *et al.* (1990)).

Autoradiographic mapping of the functional consequences of NMDA receptor blockade supports and extends the view which has emerged from behavioural studies. Non-competitive NMDA anatagonists and competitive NMDA antagonists, at doses broadly comparable in terms of anticonvulsant potency and anti-ischaemic efficacy, induce markedly dissimilar alterations in function-related glucose use in the CNS (Kurumaji *et al.*, 1989; Nehls *et al.*, 1988). Pronounced dose-related increases in glucose use were observed throughout the limbic system after non-competitive NMDA receptor antagonists with marked reduction in function-related glucose use widespread in neocortex. In contrast, the effects on glucose use of competitive NMDA receptor blockade or blockade of the glycine site are numerically small and anatomically circumscribed (Hargreaves *et al.*, 1991; Kurumaji *et al.*, 1989).

These alterations in function-related energy generation are particularly important as they appear to be predictive of the reversible morphological alterations which are observed in some brain areas after NMDA antagonists. In the rat posterior cingulate cortex, the acute administration of non-competitive NMDA antagonists, MK-801 phencyclidine and ketamine, effects a dose-dependent cellular swelling and vacuolisation, particularly in the multipolar and pyramidal medium to large sized neurones in layers III and IV. The cellular swelling and vacuolisation subsided 12 h after drug administration, and by 24 h after dizocilpine administration, the histological appearance of the tissue was essentially normal (Olney *et al.*, 1989). These reversible changes in neuronal structure are noted with MK-801 at doses (ED_{50} approx. 0.2 mg kg^{-1}) similar to those at which anti-ischaemic effects, anticonvulsant effects and increased glucose use are seen. Similar neuronal swelling and vacuolisation are also observed with competitive NMDA antagonists when administered intracerebrally and after systemic administration, although doses that are greater than those required to reduce ischaemic damage are necessary (McCulloch & Iversen, 1991).

There are a number of features that should be emphasised in relation to CNS structural changes seen after NMDA receptor blockade. First, these changes are highly circumscribed in their anatomical distribution. Secondly, neither the neuronal swelling nor the increase in glucose use are seen in the posterior cingulate cortex after repeated dizocilpine treatment. Thirdly, the metabolic activation of components of the limbic system after MK-801 and the neuronal swelling and vacuolisation response can be completely prevented by light halothane anaesthesia or centrally acting anticholinergic drugs. Fourthly, the alterations in neuronal structure are completely and rapidly (24 h) reversible (McCulloch & Iversen, 1991). Finally, the risk/benefit ratio in the clinical conditions (stroke, head trauma) in which NMDA antagonists could be used need to be considered; the occurrence of neuronal swelling in a few areas of limbic forebrain, has to be balanced against the normal outcome in stroke and head trauma – at best, significant volumes of cerebral tissue are irreversibly damaged leading to lasting disability or, at worst, the death of the patient. The use of these agents in patients at risk of brain damage is underpinned by the absence of doubt from preclinical investigations that NMDA receptor antagonists will prevent damage occurring to brain tissue in such clinical conditions if administered within a therapeutically relevant time window.

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Exhibit X (10/644,645)

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Rapid communication

Activation of metabotropic receptors has a neuroprotective effect in a rodent model of focal ischaemia

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The role of the glutamate 'metabotropic' receptor was investigated in an experimental model of focal ischaemia-induced neurodegeneration. The metabotropic agonist, trans-1-amino cyclopentane-1,3-dicarboxylic acid (t-ACPD, 20 mg/kg i.p.), was administered to mice immediately after middle cerebral artery occlusion (MCAO), which causes cerebral infarct. Seven days after MCAO, the mean infarct volume value of the t-ACPD-treated group (mean ± S.E. = 4.57 ± 0.73 mm^3) was significantly reduced, by 34.3%, compared to the vehicle-treated group (mean ± S.E. = 6.95 ± 0.59 mm^3, $P < 0.01$). This suggests that metabotropic receptor activation in the adult brain reduces excitotoxicity.

Excitatory amino acids; Metabotropic receptors; Cerebral ischaemia

There is a 'metabotropic' glutamate receptor-mediated response whose activation stimulates phosphonoinositide (PI) hydrolysis. The discovery of selective agonists such as trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) has allowed the investigation of the its functional role of this response in the brain. Since t-ACPD stimulation of PI hydrolysis is transiently activated in developing brain, i.e. during synaptogenesis, an important role of metabotropic receptors has been hypothesized in developmental neuroplasticity (Schoepp et al., 1990). In adult brain the coupling between metabotropic receptor and PI hydrolysis is usually low, i.e. poor stimulation is found. However, activation of the system becomes evident under conditions such as learning and neurodegeneration (Nicoletti et al., 1988; Seren et al., 1989). The actual in vivo significance of this activation is not yet clear. Therefore, it was decided to investigate the effect of t-ACPD in adult animals, in vivo and in a model of overactivation of metabotropic receptor function, i.e. an experimental model of neurodegeneration. Middle cerebral artery occlusion (MCAO) in mice was selected as model of neurodegeneration because of its relative similarity to the actual pathology of cerebral stroke in man.

Male CD-1 mice (28–30 g) were anaesthetized with chloral hydrate (300 mg/kg i.p.). Body temperature was maintained within physiological limits by placing the animal on a heated operating table under a dissecting microscope. Craniectomy was performed where the MCA was visible through the temporal semi-translucent surface of the skull. The dura was carefully opened and MCA was exposed and coagulated by bipolar diathermy. Immediately after surgery, the animals were given t-ACPD 20 mg/kg, or vehicle, in a volume of 10 ml/kg i.p. The mice were kept normothermic up to recovery from anaesthesia (1 h). Seven days later, the animals were anaesthetized with urethane 5% and were perfused through the heart with: (i) heparin solution (0.25 g/l) in saline for 1 min and with (ii) 10% formalin solution in sodium phosphate 0.01 N buffer pH 7.4, for 3 min. The brains were carefully removed and processed for histological staining. Five coronal sections were taken at the following brain coordinates, A2100, A2750, A3250, A4150 and A4750 um anterior to the interaural line. Sections were stained with methylene blue for image analysis. The image of the whole section was digitized and the infarct size for each section was evaluated as mm^2.

Macroscopic observation of the brains demonstrated a lack of Blue ink perfusion (10 ml/kg i.v.) in a large area of the tempoparietal cortex. Histological analysis showed a uniform and diffuse coagulation necrosis in the cortical area, clearly separated from the healthy tissue. The effect of t-ACPD 20 mg/kg i.p., given only once immediately after surgery, is shown in fig. 1. In each of the coronal brain section examined, the treatment was effective to significantly reduce infarct size

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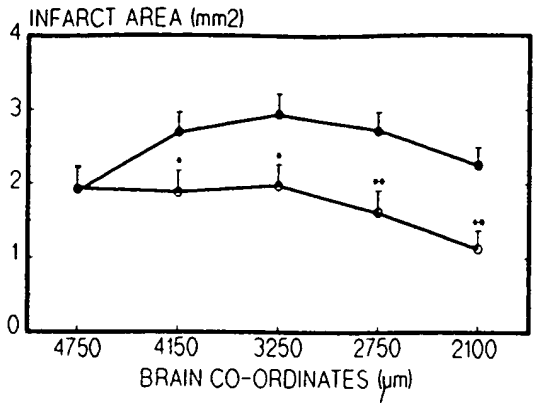

Fig. 1. Cerebral infarct area in mice treated with vehicle (•) or trans-ACPD 20 mg/kg (○) i.p., immediately after MCAO. The size of the infarct core was measured in haematoxylin-eosin-stained coronal sections taken at A2100, A2750, A3250, A4150 and A4750 μm anterior to lambda. Each point represents the mean infarct value ± S.E.M. for 17 (vehicle-treated group) and 14 (trans-ACPD group) mice. The statistical significance of differences between groups was calculated using a RS/1 (BBN Software Products Corporation) standard procedure for Student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

compared to that in the vehicle-treated group. An approximation of volume of infarct was achieved by integration of areas with the distance between each coronal section. The mean infarct volume value of the t-ACPD-treated group (mean ± S.E. = 4.57 ± 0.73 mm^3) was significantly reduced (by 34.3%) compared to the mean value calculated for the vehicle-treated group (mean ± standard error = 6.95 ± 0.59 mm^3, $P < 0.01$).

It has been reported recently that t-ACPD attenuates NMDA-induced neurotoxicity in cortical cultured cells (Koh et al., 1991). The present results confirmed and extend this observation to in vivo conditions. It is clear that the administration of a single dose of t-ACPD, given at the time of surgery, significantly inhibits neurodegeneration due to the MCAO in mice, suggesting that metabotropic receptor agonists can act as antineurodegenerative compounds. The activation of silent metabotropic receptors in neurodegeneration coupled to the present results might suggest that a possible role of metabotropic receptor activation in adult brain could be to reduce excitoxicity. The existence of more than one subtype of metabotropic receptors has been proposed recently (Vecil et al., 1991). It is difficult to establish from our results which subtype of receptor could be involved in neuroprotection. However, electrophysiological studies of striatum (Lovinger, 1991) and hippocampus (Baskys and Malenka, 1991) have indicated the existence of t-ACPD-sensitive metabotropic autoreceptors modulating glutamatergic transmission. Therefore, it could be proposed that t-ACPD was found to be neuroprotective in this study because it inhibited the excess glutamate release occurring during ischaemia via activation of these modulatory autoreceptors. Only the development of compounds selective for each specific subtype of metabotropic receptors will help to clarify this point.

Acknowledgements

We thanks Dr. A. Terron and Mrs D. Spagnolo for histological work and Dr F. Van Amsterdam for use of the image analyzer.

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Exhibit Y (10/644/645)

Neuroprotective Strategies in a Model of Chronic Glutamate-Mediated Motor Neuron Toxicity

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Abstract: A dramatic loss of glutamate transport has been observed in sporadic amyotrophic lateral sclerosis and has been postulated to contribute to the disease. Experimentally, this hypothesis was corroborated by mimicking the chronic loss of glutamate transport in postnatal rat spinal cord organotypic cultures through the use of glutamate transport inhibitors. This system is characterized by a relatively selective slow loss of ventral horn motor neurons resulting from glutamate transport inhibition. In this study, spinal cord organotypic cultures were used to test various drugs to evaluate their neuroprotective properties against this slow glutamate-mediated neurotoxicity. The most potent neuroprotectants were drugs that altered glutamate neurotransmission, including non-NMDA receptor antagonists (GYKI-52466, PD144216, and PD139977) and drugs that could block presynaptic release or synthesis (riluzole and gabapentin). In addition, some antioxidants (U83836E and *N*-*t*-butyl-α-phenylnitrone) and inhibitors of nitric oxide synthesis (N^G-monomethyl-L-arginine acetate) were modestly neuroprotective. The calcium endonuclease inhibitor aurintricarboxylic acid and the calcium release inhibitor dantrolene also provided partial motor neuron protection. However, several antioxidants and calcium channel antagonists had no excitotoxic neuroprotectant activity. This system provides a preclinical screening method for the burgeoning number of drugs postulated for clinical trials in motor neuron disease and a model to evaluate the mechanisms of chronic glutamate toxicity. **Key Words:** Excitotoxicity—Antioxidant—Glutamate transport—Motor neuron.
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Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease of motor neurons characterized neuropathologically by the slow loss of spinal and cortical motor neurons. ALS is largely a sporadic disease, with only 5–10% of cases having an autosomal dominant inheritance. Currently there are three major hypotheses that address putative causal factors for the motor neuron degeneration that occurs in ALS: (a) the autoimmune hypothesis, which suggests that antibodies to N- and L-type calcium channels could cause or contribute to the sporadic form of the disorder (Smith et al., 1992); (b) the excitatory amino acid hypothesis, which suggests that excessive synaptic glutamate, due in part to the loss of glutamate transport, or other metabolic enzymes, could contribute to motor neuron loss (Plaitakis, 1990; Rothstein et al., 1992; Shaw et al., 1994); and (c) the oxidative stress hypothesis, based on mutations in Cu/Zn superoxide dismutase, which have been found in 20% of familial ALS patients and which have been shown in transgenic animals to reproduce the disease faithfully (Rosen et al., 1993; Gurney et al., 1994). These mutations have not been observed in the sporadic ALS population (Bowling et al., 1993).

Previously, the chronic defects in glutamate transport observed in ALS have been mimicked in an organotypic culture model of long-term glutamate toxicity (Rothstein et al., 1993). Organotypic cultures of postnatal spinal cord slices have the advantages of maintaining intact morphology and local synaptic connections in culture for periods in excess of 3 months. *threo*-Hydroxyaspartate (THA) or pyrrolidone dicarboxylic acid is used to inhibit selectively glutamate transport, thereby raising extracellular glutamate concentrations. Using this paradigm, studies demonstrated that postnatal motor neurons are selectively susceptible

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Abbreviations used: *trans*-ACPD, *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; ATX, aurintricarboxylic acid; *t*-BPN, *N*-*t*-butyl-α-phenylnitrone; ChAT, choline acetyltransferase; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride; L-NMMA, N^G-monomethyl-L-arginine acetate; NOS, nitric oxide synthase; PD139977, 1*H*-benz(*g*)indole-2,3-dione-6,7,8,9-tetrahydro-5-nitro-3-oxime; THA, *threo*-hydroxyaspartate; tofisopam, 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5*H*-2,3-benzodiazepine; U83836E, (−)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol dihydrochloride.

to glutamate toxicity via non-NMDA, but not NMDA, glutamate receptors. We have used this system for preclinical screening of several compounds to assess their neuroprotective potential.

MATERIALS AND METHODS

Organotypic cultures

Organotypic cultures were prepared from lumbar spinal cord of 8-day-old rat pups as described previously (Rothstein et al., 1993; Corse and Rothstein, 1995). In brief, lumbar spinal cords were removed and sliced into 300-μm-thick dorsal-ventral sections, and five slices were placed on Millipore CM semipermeable 30-mm-diameter membrane inserts. The inserts were placed on 1 ml of culture medium in 35-mm-diameter culture wells. Culture medium consisted of 50% minimal essential medium and HEPES (25 mM), 25% heat-inactivated horse serum, and 25% Hanks' balanced salt solution (GIBCO) supplemented with D-glucose (25.6 mg/ml) and glutamine (2 mM), at a final pH of 7.2. Antibiotic and antifungal agents were not used. Cultures were incubated at 37°C in a 5% CO_2-containing humidified environment (Forma Scientific). Culture medium, along with any added pharmacological agents, was changed twice weekly.

Biochemical assays

To determine choline acetyltransferase (ChAT) activity, the spinal cord tissue in each dish (five slices) was pooled and frozen (−75°C) until assay. Each culture well represented one time point or drug concentration. ChAT activity was measured radiometrically by described methods using [^{3}H]acetyl-CoA (Amersham) (Fonnum, 1975). Protein content of tissue homogenates was determined by a Coomassie Protein Assay kit (Pierce, Rockford, IL, U.S.A.).

Chronic toxicity model

For all experiments, cultures were used 8 days after preparation, at which time THA (100 or 500 μM), test drug alone, or THA along with test drug were added to culture medium. For each new drug studied, these controls (untreated cultures, test drug alone, and THA-treated cultures) were always included in the experimental design. Putative neuroprotective drugs were tested in cultures that were incubated for either 14 days with 500 μM THA ("high toxicity") or 28 days with 100 μM THA ("low toxicity"). At the end of this period, cultures were collected and assayed for ChAT activity. Because of the chronic nature of these experiments and the paucity of data on chronic administration of the various drugs studied, all drugs were used at concentrations typically lower than those used in previously published acute studies.

Statistics

Analysis of neuroprotective effect was performed by comparison of drug treatments in the presence of THA compared with the THA effect alone by one-way ANOVA. Subsequent statistical evaluations were performed by Student's t test using the Bonferroni correction. Because neuroprotection was studied using either high or low dose THA, as a means of comparing the relative neuroprotection, an additional analysis of efficacy of neuroprotection was performed by normalizing data using the following formula: % neuroprotection = [(drug effect as % untreated control) − (THA effect as % untreated control)] × [100/(100 − THA effect as % untreated control)]. Drug effect was defined as culture ChAT activity in cultures treated with both THA and neuroprotective drug. Untreated control was defined as the ChAT activity of untreated cultures. THA effect was defined as ChAT activity of cultures in the presence of THA. In that formula, THA effect as a percentage of the untreated control was 21.9% using 500 μM THA and 34.4% using 100 μM THA.

Materials

1H-Benz(g)indole-2,3-dione-6,7,8,9-tetrahydro-5-nitro-3-oxime [PD139977; identical to NS 102 (Neurosearch, Denmark)], PD144216 [identical to NS 257 (Neurosearch)], phenytoin, and gabapentin were kindly provided by Dr. Charles Taylor (Parke-Davis, Ann Arbor, MI, U.S.A.). 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI-52466) was obtained from Istvàn Tarnawa (Institute for Drug Research, Budapest, Hungary). Tofisopam [1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine] was obtained from Egis Pharmaceuticals (Budapest). Riluzole was provided by Rhone-Poulenc Rorer (Paris, France). Cyclothiazide was provided by Eli Lilly and Co (Indianapolis, IN, U.S.A.). Ceftriaxone was provided by Hoffmann-La Roche (Nutley, NJ, U.S.A.). NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (*trans*-ACPD), N^G-monomethyl-L-arginine acetate (L-NMMA) were all obtained from Research Biochemicals International (Natick, MA, U.S.A.). (−)-2-[[4-(2,6-Di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol dihydrochloride (U83836E) was kindly provided by Dr. E. Hall (Upjohn Co., Kalamazoo, MI, U.S.A.). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Two levels of chronic glutamate toxicity were studied, depending on the presumed mechanisms or potency of the putative neuroprotective agent. "High-toxicity" treatment with 500 μM THA reliably produced ~80% loss of ChAT activity after only 2 weeks of treatment, whereas "low-toxicity" THA (100 μM) produced ~65% loss of ChAT activity after only 4 weeks of treatment (Table 1). The loss of ChAT activity has been shown previously to correlate with, and therefore to reflect quantitatively, the loss of lumbar spinal motor neurons in this culture paradigm (Rothstein et al., 1993). "High-toxicity" THA produces a persistent elevation in the concentration of extracellular glutamate of ~25 μM, whereas "low-toxicity" THA produces a chronic elevation in the concentration of extracellular glutamate of ~3–5 μM (Rothstein et al., 1993).

The drugs studied could be roughly classified into the following groups: non-NMDA receptor antagonists and allosteric modulators [GYKI-52466, cyclothiazide (Yamada and Tang, 1993; Zorumski et al., 1993), PD144216, and PD139977 (Johansen et al., 1993)];

TABLE 1. *Effects of various putative neuroprotectants on chronic glutamate neurotoxicity*

Uptake inhibitor	Weeks of treatment	Mechanism or site of action	Putative neuroprotectant (μM)	% of untreated control	p
THA (500 μM)	2		—	20.9 ± 1.6	—
		Glutamate receptor/ release	GYKI-52466 (100)	96.0 ± 5.0	<0.01
			Cyclothiazide (100)	0.5 ± 0.5	<0.01
			trans-ACPD (50)	22.0 ± 13.0	NS
			Riluzole (10)	27.2 ± 5.7	NS
			Riluzole (100)	51.7 ± 6.7	<0.01
		Ion channels	Glibenclamide (100)	55.4 ± 9.7	<0.01
			Dantrolene (3)	22.0 ± 3.7	NS
			Dantrolene (30)	44.2 ± 10.0	<0.05
			Bepridil (50)	23.0 ± 2.0	NS
			Nifedipine (1)	16.0 ± 3.0	NS
			Nifedipine (10)	17.4 ± 2.2	NS
			Ethacrynic acid (100)	27.5 ± 3.5	NS
		Antioxidant	*t*-BPN (250)	36.5 ± 0.7	<0.01
			U83836E (100)	34.3 ± 8.5	<0.05
			Vitamin E (100)	19.5 ± 4.0	NS
			Ascorbic acid (1,000)	19.0 ± 4.0	NS
		Other	ATX (100)	30.8 ± 2.2	<0.01
			Tofisopam (100)	19.0 ± 4.0	NS
THA (100 μM)	4		—	34.4 ± 5.3	—
		Glutamate receptor/ synthesis	PD144216 (10)	70.0 ± 5.0	<0.01
			PD139977 (10)	63.0 ± 5.0	<0.05
			Gabapentin (10)	40.0 ± 12.0	NS
			Gabapentin (33)	43.2 ± 2.4	NS
			Gabapentin (100)	100.0 ± 21.3	<0.01
		Ion channel	Phenytoin (100)	39.0 ± 9.0	NS
		Antioxidant	L-NMMA (150)	61.0 ± 12.7	<0.05
			Mannitol (1,000)	33.0 ± 9.8	NS
		Other	Ceftriaxone (1)	45.0 ± 10.0	NS
			Ceftriaxone (100)	66.0 ± 8.0	<0.05

Spinal cord organotypic cultures were incubated for 2 weeks in the presence of 500 μM THA and the indicated drugs or for 4 weeks with 100 μM THA and the indicated drugs. Data are expressed as mean ± SE percentages (n = 3–6 experiments) of values from untreated control cultures. Each experiment represents the mean ChAT activity from three to five culture wells, each containing five spinal cord slices. Statistical comparisons were performed by comparing drug treatment and appropriate THA-treated cultures using *t* tests. NS, not significant.

metabotropic glutamate receptor agonists (*trans*-ACPD); ionotropic glutamate receptor agonists (AMPA, kainate, and NMDA); sodium/potassium channel inhibitors (glibenclamide, phenytoin, and riluzole); antioxidants [(+)-α-tocopherol (vitamin E), ascorbic acid (vitamin C), *N*-*t*-butyl-α-phenylnitrone (*t*-BPN), mannitol, U83836E, and L-NMMA]; and calcium release/channel inhibitors (dantrolene, nifedipine, and bepridil). Other drugs included the chloride channel inhibitor ethacrynic acid, the calcium endonuclease inhibitor aurintricarboxylic acid (ATX), the benzodiazepine agonist diazepam, the anticonvulsant gabapentin, the cephalosporin antibiotic ceftriaxone, and the GYKI-52466 analogue tofisopam. Overall, multiple drugs were found to be significantly neuroprotective ($p < 0.001$) by ANOVA (Table 1). None of these drugs demonstrated significant intrinsic neurotoxicity when maintained at the indicated concentrations (Table 1) for 2–4 weeks in culture. The chelating agent desferrioxamine (100 μM) was tested in this system but had intrinsic neurotoxicity when maintained for 2–4 weeks in culture. For comparison, data on GYKI-52466, NMDA and AMPA, published previously (Rothstein et al., 1993), are included in Tables 1 and 2.

Motor neurons were highly sensitive to direct appli-

TABLE 2. *Effect of glutamate receptor agonists on motor neuron survival*

Agonist	Concentration (μM)	% of untreated control
AMPA	10	53.0 ± 4.0
	100	26.0 ± 3.0
Kainate	1	103.0 ± 12.0
	10	32.0 ± 6.0
NMDA	10	94.0 ± 12.0
	100	96.0 ± 12.0

Drugs were added to culture medium and maintained chronically for 7 days. ChAT activity in treated cultures was compared with that in untreated controls. Data are mean ± SE values from three to five culture wells, each containing five spinal cord slices.

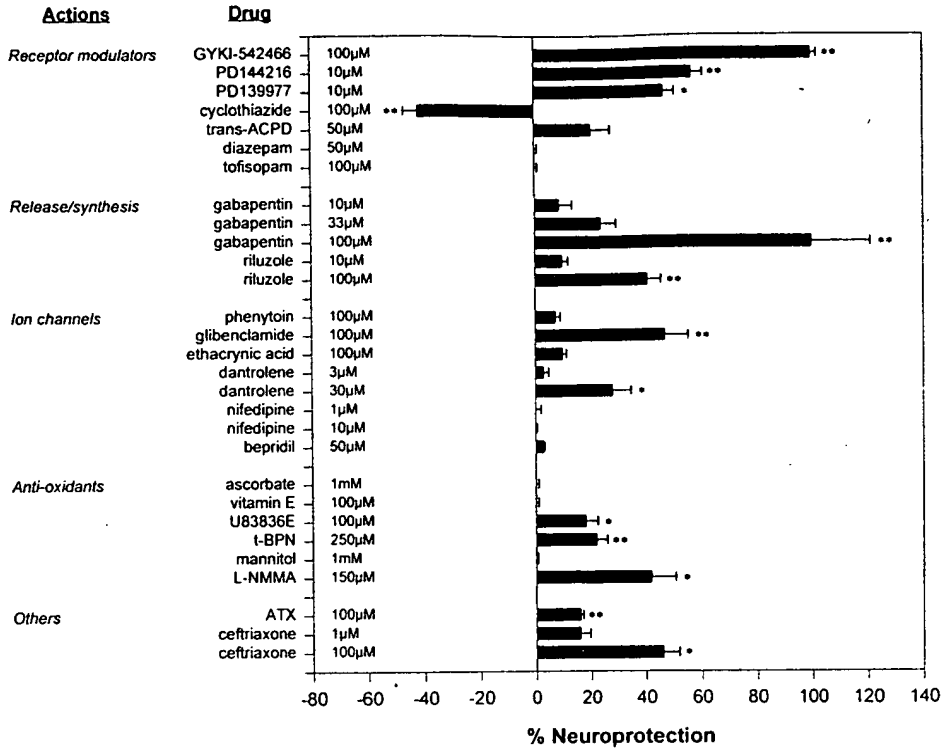

FIG. 1. Comparison of the relative neuroprotective properties of all drugs studied. Percent neuroprotection was calculated on the ability of a putative neuroprotectant to prevent THA-induced toxicity, relative to the toxicity of THA toxicity itself. In this manner, the neuroprotection of "high toxicity" and "low toxicity" can be compared. Data are mean ± SE (bars) values. Values were calculated using the formula described in Materials and Methods. Statistically significant differences between drug treatment and appropriate THA-treated cultures, as shown in Table 1, are indicated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.001$.

cation of the non-NMDA agonists kainate and AMPA (Table 2) but not to NMDA. As shown in Table 1, the motor neuron toxicity associated with chronic high- or low-dose THA could be minimized best with non-NMDA antagonists (GYKI-52466, PD144216, and PD139977), whereas the non-NMDA allosteric modulator cyclothiazide potentiated THA-mediated toxicity.

The second most effective drug was gabapentin, which almost completely protected against 100 μM THA at high, but not low, doses. Other agents that possessed significant neuroprotective properties included high-, but not low-, dose riluzole, ceftriaxone, and dantrolene. Several chemicals that can act directly or indirectly as antioxidants also partially protected against glutamate-induced motor neuron toxicity: the nitric oxide synthetase (NOS) inhibitor L-NMMA, the spin trap reagent *t*-BPN, and the vitamin E derivative U83836E. However, not all antioxidants were neuroprotective, including mannitol, vitamin E, and ascorbate.

To compare the efficacy of drugs under different toxicity paradigms, that is, high- or low-THA toxicity, the percent neuroprotection of each drug was calculated (Fig. 1). Drugs that act to block non-NMDA glutamate receptors (GYKI-52466, PD144216, and PD139977) or alter glutamate synthesis (gabapentin) were the most neuroprotective. Several were not able to protect against chronic glutamate toxicity, including tofisopam, nifedipine, bepridil, mannitol, vitamin E, ascorbic acid, ethacrynic acid, and phenytoin (Table 1 and Fig. 1).

DISCUSSION

Human and animal studies suggest chronic glutamate toxicity could cause, or at least participate in, the slow loss of motor neurons characteristic of ALS (Rosen et al., 1993; Rothstein et al., 1993). Increases in extracellular (serum or CSF) glutamate concentrations have been observed in some (Plaitakis and Caroscio, 1987; Rothstein et al., 1990; Camu et al., 1993) but not all (Perry et al., 1990; Camu et al., 1993) studies. A regional loss of high-affinity, sodium-dependent glutamate transport has also been found in brain membranes from sporadic ALS patients (Rothstein et al., 1992; Shaw et al., 1994), and it now appears that a specific astroglial subtype (GLT-1) of glutamate transporters is responsible (Jin et al., 1994). Mimicking this defect in organotypic spinal cord cultures leads to a relatively selective, slow loss of motor neurons

(Rothstein et al., 1993). Thus, the loss of glutamate transport in ALS could lead to chronically elevated glutamate levels, which either cause or contribute to the slow loss of motor neurons characteristic of this disorder. Furthermore, chronic glutamate toxicity could interact with other neurotoxic mechanisms such as oxygen radical-mediated toxicity, nitric oxide toxicity, calcium excess, energy failure, or age-related mitochondrial depletion. For example, oxygen radical damage to cells, which can damage the glutamate transporter (Volterra et al., 1994), could be secondarily amplified by subsequent glutamate toxicity. Therefore, an experimental model that could be used to evaluate the neuroprotective properties of multiple classes of compounds may have broad implications. We have developed a chronic glutamate toxicity model in spinal cord organotypic cultures for this purpose. This model offers the advantage of preserving (a) local synaptic connections, (b) morphologically intact motor neurons, and (c) glutamate receptor and transporter distributions similar to those in vivo (authors' unpublished data) for >3 months in culture.

There are many possible ways to disrupt the neurotoxic effects of glutamate: inhibit presynaptic release, inhibit neurotransmitter synthesis, block postsynaptic receptors, or interfere with the various neurotoxic cellular processes that glutamate may induce, such as formation of oxygen radicals or calcium release. Motor neurons in organotypic spinal cord cultures (Rothstein et al., 1993) and in other spinal cord neuron studies (Fletcher et al., 1988; Stewart et al., 1991; Wang et al., 1991; Couratier et al., 1994) have been found to be selectively susceptible to glutamate-mediated toxicity only via the non-NMDA receptor, and accordingly only non-NMDA antagonists such as 6-cyano-7-nitroquinoxaline-2,3-dione and GYKI-52466 were found to be neuroprotective (Rothstein et al., 1993). Agents that could potently interfere with the synthesis of neurotransmitter glutamate, such as methionine sulfoximine, or that could interfere with presynaptic release, such as tetrodotoxin (Tasker et al., 1992), were also quite protective (Rothstein et al., 1993). Thus, this culture paradigm is unique in that it provides a system for easily studying non–NMDA-mediated toxicity. It is important that immature spinal neuron culture paradigms (Mentis et al., 1993) and some (Yum and Faden, 1990; Bakshi et al., 1992; Long et al., 1994), but not all, in vivo studies have observed motor neuron sensitivity to NMDA-mediated toxicity. These differences could be the result of species differences, age of the animals studied, and, finally, the possibility of motor neuron selection bias in long-term cultures. In spite of these possible shortcomings, the organotypic spinal cord culture system is unique in providing a means to study motor neurons conveniently and efficaciously under chronic conditions.

In the current study, we sought to extend the evaluation of neuroprotective agents to include also those that might become clinically relevant, such as PD139977, PD144216, U83836E, gabapentin, and riluzole, and to use these drugs to explore the mechanisms by which chronic glutamate exposure can be neurotoxic. For several reasons, GYKI-52466, methionine sulfoximine, and tetrodotoxin are not suitable for clinical use. Although some drugs have been shown to block glutamate toxicity under acute conditions, this does not guarantee their usefulness under conditions of chronic administration, for example, chronic administration could be cytotoxic.

Glutamate receptors

Motor neurons in this organotypic spinal cord culture demonstrate sensitivity to glutamate toxicity selectively via non-NMDA receptors as demonstrated by the sensitivity of the cultures to direct application of AMPA and kainate but not to NMDA (Table 2). This is supported by potent neuroprotection by the competitive non-NMDA antagonists PD144216, PD139977, and 6-cyano-7-nitroquinoxaline-2,3-dione (Rothstein et al., 1994) as well as by GYKI-52466 (Table 1). Previously, NMDA antagonists were shown to be ineffective at protecting motor neurons from chronic glutamate toxicity (Rothstein et al., 1993).

GYKI-52466 and cyclothiazide have been recently shown to have opposing actions as allosteric modulators of the AMPA receptor (Donevan and Rogawski, 1993; Yamada and Tang, 1993; Zorumski et al., 1993). Cyclothiazide potentiated glutamate-induced motor neuron toxicity, and this is likely due to the fact that it has been shown to reduce desensitization greatly and to prolong the duration of excitatory postsynaptic currents (Yamada and Tang, 1993; Zorumski et al., 1993). Tofisopam, which is a 2,3-benzodiazepine structurally related to GYKI-52466, had no neuroprotective properties.

Glutamate synthesis and release

Agents that could block presynaptic release were found to be partially protective. Riluzole reportedly acts to block voltage-activated sodium channels and to inhibit presynaptic glutamate release (Drejer et al., 1986; Doble et al., 1992). A recent study suggested riluzole apparently increased survival in a small subset of ALS patients with bulbar onset (Bensimon et al., 1994). The organotypic culture data suggest further that riluzole's interference with glutamate actions can be motor neuron protective.

Gabapentin is a new anticonvulsant, whose mechanism of action, at the cellular level, is not yet fully understood. Recently, gabapentin has been found to inhibit branched-chain amino acid transport and branched-chain amino acid aminotransferase (Stewart et al., 1993; Su et al., 1994). Branched-chain amino acid aminotransferase leads to the formation of glutamate, and it has been hypothesized that gabapentin might act to decrease neurotransmitter glutamate synthesis (Taylor, 1993). Regardless of its mechanism of

action, gabapentin was able to protect in our model against glutamate toxicity, and this occurred at clinically relevant concentrations (D. Welty, Parke-Davis, personal communication). Both riluzole and gabapentin are currently in clinical trials for ALS.

It is interesting that glibenclamide was also neuroprotective. Glibenclamide, a sulfonylurea, is an inhibitor of ATP-sensitive K^+ channels (K_{ATP}) and is from a class of drugs commonly used as antihyperglycemics. Under normal conditions or conditions of anoxia/ischemia, sulfonylureas can act acutely to increase GABA and glutamate release (Amoroso et al., 1990; Zini et al., 1993). Although ATP-dependent K^+ channels have been localized to brain (Bernardi et al., 1988; Mourre et al., 1989), their role in normal neurochemistry is not well understood. It could be that chronic glibenclamide could act to protect against toxicity by, in part, stimulating the glycolytic pathway and thereby inhibiting a depletion of ATP (Edwards and Weston, 1993). It is not yet known whether glibenclamide can act at the glutamate transporter to stimulate transport or interfere with inhibitors of glutamate transport.

Ceftriaxone, a cephalosporin antibiotic, was also mildly neuroprotective. The mechanism of neuroprotection for this agent is not known. It is interesting that the structural nucleus of cephalosporins contains a side chain composed of D-α-aminoadipic acid (Mandell and Sande, 1990). D-α-Aminoadipic acid can act as an NMDA antagonist and can also decrease non–NMDA-mediated toxicity (McBean, 1990). However, it is not known whether ceftriaxone, or its degradation products, can act as a non-NMDA antagonist or in some other way alter glutamatergic neurotransmission.

The molecular mechanism by which glutamate causes *chronic* neurotoxicity is not known, but by extrapolating from what is known about the actions of glutamate under acute conditions (Choi, 1992), especially through its actions at non-NMDA receptors, several neurotoxic pathways are possible. For example, activation of non-NMDA receptors can stimulate xanthine oxidase (Dykens, 1994), leading to increased superoxide formation. Excessive chronic depolarization could alter cellular ATP levels or could activate ion channels, such as the Na^+-Ca^{2+} exchanger, to balance increased intracellular sodium concentrations. Therefore, we sought to evaluate agents that could interfere with free radical generation or abnormal calcium homeostasis.

Calcium channels

Intraneuronal calcium levels may be increased by several mechanisms. Following stimulation of glutamate receptors, an increase in intracellular calcium content is believed to participate in glutamate-mediated neurotoxicity (Garthwaite and Garthwaite, 1986; Hartley and Choi, 1989; Manev et al., 1989). In some cases, calcium may leak directly through the channel leading to activation of calcium-dependent toxic intracellular pathways. This is the case in the NMDA receptor and in non-NMDA receptors lacking the GluR2 subunit (Hollmann et al., 1991). Intracellular calcium content can also be increased via the Na^+-Ca^{2+} membrane exchanger, through various voltage-dependent calcium channels, or via intracellular stores such as mitochondria (for review, see Frandsen and Schousboe, 1993). From our studies it appears that the calcium channel antagonists bepridil and nifedipine were ineffective in preventing the neurotoxicity associated with chronic glutamate exposure, although dantrolene, which has been shown to protect against NMDA-mediated toxicity in cerebellar granule cells (Frandsen and Schousboe, 1992), was slightly protective. Unlike voltage-activated calcium channels or the Na^+-Ca^{2+} exchanger, dantrolene acts in muscle to inhibit calcium release from intracellular sarcoplasmic reticulum stores. In neurons, the increase in intracellular calcium content caused by glutamate can be blocked by dantrolene's actions (Frandsen and Schousboe, 1992, 1993). Previous studies of cortical neurons have suggested that dantrolene could block quisqualate-, glutamate-, or NMDA-induced rises of intracellular calcium level but not those induced by kainate or AMPA (Frandsen and Schousboe, 1992). Because motor neurons are unique in their non-NMDA receptor sensitivity, it may be that, unlike cultured cortical neurons, dantrolene could block intracellular calcium release in organotypic motor neurons following non-NMDA receptor stimulation.

Oxidative stress and nitric oxide

The direct free radical scavengers *t*-BPN and U83836E were also neuroprotective, strongly suggesting that chronic formation of radical species contributes to the toxicity. It has been suggested that glutamate action at non-NMDA receptors, which has been shown to activate xanthine oxidase (thereby increasing levels of oxygen radicals), could act to target motor neurons for selective degeneration (McNamara and Fridovich, 1993). Several other studies have demonstrated that glutamate, at least acutely, can lead to the formation of free radicals via xanthine oxidase activation of non-NMDA receptors (Dykens, 1994), release of arachidonic acid (Lazarewicz et al., 1988), or the formation of nitric oxide (Garthwaite et al., 1989; also see the review of Coyle and Puttfarcken, 1993). Compounding this direct toxicity, oxygen radicals, free fatty acids, and arachidonic acid can perpetuate this toxicity by inactivating glutamate transport (Chan et al., 1983; Rhoads et al., 1983; Barbour et al., 1989; Volterra et al., 1992, 1994), thereby initiating a possible vicious cycle.

The NOS inhibitor L-NMMA was also neuroprotective, but the mechanisms by which such drugs could protect motor neurons is not understood. Motor neurons do not possess NOS under normal conditions (Dun et al., 1992) but can express NOS following

injury (Wu, 1993; Yu, 1994). It is interesting that NOS inhibitors have been found to protect against motor neuron death in a different insult, spinal root avulsion (Wu and Li, 1993). In organotypic spinal cord cultures, it may be that small NOS-positive neurons surrounding motor neurons produce neurotoxic nitric oxide, which diffuses locally to cause, or to contribute to, glutamate toxicity. Alternatively, chronic elevated glutamate levels could lead to direct or indirect induction of motor neuron NOS, which could potentiate glutamate toxicity.

Apoptosis

Recently, acute and chronic oxidative stress was found to produce apoptotic cellular degeneration in embryonic neurons (Ratan et al., 1994) and postnatal organotypic spinal cord cultures (Rothstein et al., 1994). Although there are likely to be multiple pathways that produce the morphological appearance of apoptosis, in some experimental paradigms activation of calcium endonuclease is a critical step in the DNA degradation that occurs. ATX, an inhibitor of calcium endonuclease, has been shown to block apoptotic cell death (Batistatou and Greene, 1991) and possibly glutamate-mediated neurotoxicity (Roberts-Lewis et al., 1993; Samples and Dubinsky, 1993). Although ATX can also block the actions of glutamate at the NMDA receptor in some systems (Zeevalk et al., 1993), this pharmacological property is irrelevant in the organotypic spinal cord model because glutamate-mediated motor neuron toxicity occurs only via non-NMDA receptors (Rothstein et al., 1993). The small but significant neuroprotection afforded by ATX under conditions of glutamate toxicity may reflect the apoptotic cell death that can occur in association with prolonged glutamate exposure. Detailed evaluation of organotypic cultures reveals that, although the majority of cell death appears necrotic, increased apoptotic neurons are present compared with controls (authors' unpublished data).

In summary, inefficient glutamate transport can be mimicked in organotypic spinal cord cultures by chronic blockade of glutamate transport. Using this paradigm, various drugs can be readily evaluated for their neuroprotective properties. From these studies we have learned that several chemicals appear to be protective and might be considered for future clinical investigation. Furthermore, it may be that the mechanisms of chronic glutamate toxicity are likely to be multifactorial, involving not only glutamate release, glutamate synthesis, and non-NMDA glutamate receptor actions, but also oxidative injury, altered calcium release, and activation of calcium endonuclease.

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Exhibit 2 (10/644,645)

Cellular Mechanisms of Protection by Metabotropic Glutamate Receptors During Anoxia and Nitric Oxide Toxicity

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Abstract: Metabotropic glutamate receptors, nitric oxide (NO), and the signal transduction pathways of protein kinase C (PKC) and protein kinase A (PKA) can independently alter ischemic-induced neuronal cell death. We therefore examined whether the protective effects of metabotropic glutamate receptors during anoxia and NO toxicity were mediated through the cellular pathways of PKC or PKA in primary hippocampal neurons. Pretreatment with the metabotropic glutamate receptor agonists (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), and L(+)-2-amino-4-phosphonobutyric acid (L-AP4) 1 h before anoxia or NO exposure increased hippocampal neuronal cell survival from ~30 to 70%. In addition, posttreatment with 1*S*,3*R*-ACPD or L-AP4 up to 6 h following an insult attenuated anoxic- or NO-induced neurodegeneration. In contrast, treatment with L-(+)-2-amino-3-phosphonopropionic acid, an antagonist of the metabotropic glutamate receptor, did not significantly alter neuronal survival during anoxia or NO exposure. Protection by the ACPD-sensitive metabotropic receptors, such as the subtypes mGluR1α, mGluR2, and mGluR5, appears to be dependent on the modulation of PKC activity. In contrast, L-AP4-sensitive metabotropic glutamate receptors, such as the subtype mGluR4, may increase neuronal survival through PKA rather than PKC. Thus, activation of specific metabotropic glutamate receptors is protective during anoxia and NO toxicity, but the signal transduction pathways mediating protection differ among the metabotropic glutamate receptor subtypes. **Key Words:** Anoxia—Hippocampal neurons—Metabotropic glutamate receptor—Nitric oxide—Protein kinase A—Protein kinase C.
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Both neuronal networks and neuronal transmitters have been shown to modulate neuronal plasticity and neuronal survival (Maiese et al., 1992, 1994*a*; Nellgard and Wieloch, 1992). More recently, the nine cloned metabotropic receptor subtypes (mGluR1α, mGluR1β, mGluR1c, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, and mGluR7) have been linked to the modulation of neuronal survival. They function through several signal transduction pathways, such as cyclic AMP (cAMP), protein kinase C (PKC), inositol phosphate, ion channel flux, and phospholipase D. Activation of the metabotropic receptors can reduce *N*-methyl-D-aspartate (NMDA) toxicity in retinal cells (Siliprandi et al., 1992) and in cortical neurons (Koh et al., 1991*a*), lessen epileptiform activity in the rat cortex (Sheardown, 1992), protect synaptic transmission during periods of hypoxia (Opitz and Reymann, 1991, 1993), and increase neuronal survival during nitric oxide (NO) exposure (Maiese et al., 1995). Although in some experimental models the activation of metabotropic receptors may appear to be deleterious (Sacaan and Schoepp, 1992), several studies support the premise that activation of the various metabotropic receptor subtypes in the hippocampus can depress rather than excite synaptic transmission and can function to decrease high-voltage-activated calcium currents in the hippocampus (Swartz and Bean, 1992; Sahara and Westbrook, 1993; Gereau and Conn, 1995).

The mechanisms that mediate neuroprotection by this group of receptors during ischemic disease are not clear. During cerebral ischemia, glutamate receptor activation can lead to both calcium influx into neurons and the production of NO (Garthwaite et al., 1989). NO synthase (NOS) is known to be induced in hippo-

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Abbreviations used: 1*S*,3*R*-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; *trans*-ACPD, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid; L-AP3, L(+)-2-amino-3-phosphonopropionic acid; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; cAMP, cyclic AMP; dbcAMP, dibutyryladenosine 3′,5′-cyclic monophosphate; NO, nitric oxide; NOS, nitric oxide synthase; PDD, 4-phorbol 12,13-didecanoate; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SNP, sodium nitroprusside.

campal astrocytes during this period (Endoh et al., 1994) with the subsequent generation of NO in the cerebral cortex (Sato et al., 1993). In addition, glutamate receptors have been reported to stimulate directly NO production in neurons (Marin et al., 1993). Inhibition of NO production in vitro during glutamate toxicity (Dawson et al., 1991) or during anoxia (Maiese et al., 1993*a*; Maiese and Boccone, 1995) is protective against neuronal cell death. Thus, changes in metabotropic glutamate receptor activity, such as in the hippocampus (Fotuhi et al., 1994), may represent one of the pathways that influences anoxic neuronal cell death and NO toxicity during cerebral ischemia.

Neuroprotection via the metabotropic glutamate receptors also may be mediated through specific signal transduction pathways. Metabotropic glutamate receptor function may require the activation of second messengers such as PKC (Koh et al., 1991*a*). PKC also can function as a feedback mechanism on metabotropic glutamate activity and can reduce the inhibitory effects of this receptor on excitatory transmission at corticostriatal synapses (Swartz et al., 1993). PKC activation independently has been linked to ischemic neurodegeneration. Antagonism of PKC activity has been shown to reduce neuronal death during toxicity associated with glutamate, kainate, anoxia, and NO (Favaron et al., 1988; Maiese et al., 1993*b*,*c*; Maiese and Boccone, 1995).

Modulation of protein kinase A (PKA) activity may mediate the neuroprotective ability of metabotropic receptor activation during ischemia. Some metabotropic glutamate receptor subtypes, such as those responsive to (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), have been shown to increase cAMP levels (Winder and Conn, 1993). Activation of PKA can be neuroprotective. For example, pharmacological activators of PKA, like platelet-derived growth factor, can protect fibroblasts from the toxic effects of serum deprivation (Tamm and Kikuchi, 1991), and increased PKA activity increases neuronal survival during NO toxicity (Maiese et al., 1993*b*).

Thus, we examined whether metabotropic glutamate receptor activity modulated neuronal survival during anoxia and NO toxicity and whether this modulation was dependent on the PKC and PKA pathways. We focused on the ACPD- and L(+)-2-amino-4-phosphonobutyric acid (L-AP4)-sensitive metabotropic glutamate receptors. These receptors have previously been linked to neuroprotection during cerebral ischemia (Chiamulera et al., 1992), NMDA toxicity (Koh et al., 1991*a*), and NO exposure (Maiese et al., 1995) and have been associated with the inhibition of calcium currents (Trombley and Westbrook, 1992), which could subsequently alter the production of NO (Maiese et al., 1994*b*). We demonstrate that activation of metabotropic glutamate receptors protects hippocampal neurons from anoxia and NO toxicity and that the mechanism of protection by these receptors may involve modulation of both PKC and PKA.

MATERIALS AND METHODS

Hippocampal neuronal cultures

The hippocampi were obtained from 1-day-old Sprague–Dawley rat pups and incubated in dissociation medium (90 m*M* Na_2SO_4, 30 m*M* K_2SO_4, 5.8 m*M* $MgCl_2$, 0.25 m*M* $CaCl_2$, 10 m*M* kynurenic acid, and 1 m*M* HEPES with the pH adjusted to 7.4) containing papain (10 U/ml) and cysteine (3 mmol/L) for two 20-min periods. The hippocampi were then rinsed in dissociation medium and incubated in dissociation medium containing trypsin inhibitor (10–20 U/ml) for three times, 5 min each. The cells were washed in growth medium: Leibovitz's L-15 medium (GibcoBRL, Gaithersburg, MD, U.S.A.) with 6% sterile rat serum (Bioproducts for Science, Indianapolis, IN, U.S.A.), 150 m*M* $NaHCO_3$, 2.25 mg/ml of transferrin, 2.5 μg/ml of insulin, 10 n*M* progesterone, 90 μ*M* putrescine, 15 n*M* selenium, 35 m*M* glucose, 1 m*M* L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) and supplemented with vitamins. The dissociated cells were plated at a density of ~1.5 × 10^6 cells/mm^2 in 35-mm-diameter polylysine/laminin-coated plates (Falcon Labware, Lincoln Park, NJ, U.S.A.). Neurons were maintained in growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% room air. All experiments were performed with neurons that had been in culture for 2–3 weeks. Nonneuronal cells accounted for <20% of the total cell population.

Experimental treatments

Cultures were deprived of oxygen by placing them in a humidified atmosphere at 37°C with 95% N_2 and 5% CO_2 for an 8-h period. The cultures are maintained in an anaerobic environment for the entire 8-h period with oxygen tension maintained at 0% and monitored within ±0.1% of the selected set point. NO administration was performed by replacing the culture media with media containing 300 μ*M* sodium nitroprusside (SNP; Sigma Chemical Co., St. Louis, MO, U.S.A.) for 5–10 min. At this dose of SNP, ~70–80% of the neurons are killed by a mechanism that involves generation of NO. In our previous work (Maiese et al., 1993*a*), we have demonstrated that SNP toxicity in hippocampal neuronal cultures is directly linked to the generation of NO. Other metabolic products of SNP, such as potassium cyanide, require prolonged administration at elevated doses to achieve the toxicity generated by SNP or other NO generators. Following treatment with oxygen deprivation or NO, culture medium was replaced with fresh growth medium, and the cultures were placed in a normoxic, humidified incubator at 37°C with 5% CO_2 for 24 h before assessing cell death.

The metabotropic receptor ligands L(+)-2-amino-3-phosphonopropionic acid (L-AP3), L-AP4, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD), and 1*S*,3*R*-ACPD were obtained from Tocris Neuramin (Bristol, U.K.). All agents were added directly to the cultures 1 h before anoxia or NO exposure or during the specified post-treatment paradigms.

The pharmacological agents dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP), phorbol 12-myristate 13-acetate (PMA), and 4-phorbol 12,13-didecanoate (PDD) were obtained from Sigma Chemical Co. The drugs H-7 and H-89 were purchased from Calbiochem Co. (La Jolla, CA, U.S.A.). All agents were added directly to the cultures 1 h before anoxia or NO administration with the exception of PMA (1 μ*M*) and PDD (1 μ*M*), which required a 24-h

pretreatment before anoxia or NO administration. Experimental cultures received replacement of the pharmacological agents during any growth media changes.

Assessment of neuronal cell death

Hippocampal neuronal injury was determined by bright-field microscopy using a 0.4% trypan blue dye exclusion method 24 h following treatment with anoxia or SNP. Neurons were identified by morphology. The mean survival was determined by counting eight randomly selected nonoverlapping fields without astrocyte growth containing ~10–20 neurons (viable plus nonviable) in each 35-mm-diameter Petri dish. The area of each sampled field was 0.80 mm^2, and the combined eight fields selected represent ~8% of the neuronal population in each Petri dish. The mean survival from each culture dish represents an n = 1 determination. Each experiment was replicated six times independently on separate occasions with different cultures.

Statistical analysis

Results of neuronal survival among the different cell culture groups during exposure to anoxia or SNP were considered to be statistically different with $p < 0.001$, by ANOVA and unpaired Student's t test.

RESULTS

Pharmacological activation of ACPD-sensitive metabotropic receptors protects hippocampal neurons from anoxia and NO toxicity

We initially chose to evaluate the role of ACPD-sensitive metabotropic receptor subtypes, such as mGluR1α, mGluR2, and mGluR5, with the ligands *trans*-ACPD and the more selective agonist 1*S*,3*R*-ACPD (Schoepp et al., 1991) during anoxia and NO toxicity. One hour before exposure to anoxia or SNP (300 μM), we administered increasing doses of *trans*-ACPD or 1*S*,3*R*-ACPD (10, 30, 100, 250, 500, 750, and 1,000 μM). Consistent with prior work evaluating the effects of *trans*-ACPD on cortical neurons (Koh et al., 1991*b*), *trans*-ACPD and 1*S*,3*R*-ACPD were not toxic to the hippocampal neurons, even at concentrations of 1,000 μM (Fig. 1A and B). Increasing concentrations of *trans*-ACPD were neuroprotective against anoxia and NO toxicity, and the most significant effects occurred in the range of 250–1,000 μM with neuronal survival increasing to ~75% (Fig. 1A). To a similar degree, 1*S*,3*R*-ACPD also was protective against anoxia and exposure to SNP. With this group of hippocampal cultures, survival increased from ~30% to a maximum of ~80% (Fig. 1B).

An agonist of the metabotropic receptor mGluR4 increases neuronal survival during anoxia and NO toxicity

In this set of experiments, we evaluated the neuronal metabotropic receptor mGluR4 in hippocampal neurons because the receptor can be pharmacologically isolated with the potent agonist L-AP4 (Schulte et al., 1992). The ligand L-AP4 was administered 1 h before initiation of anoxia or exposure to SNP. L-AP4 was not toxic to the hippocampal neurons at any of the concentrations administered (10, 30, 100, 250, 500, 750, and 1,000 μM; Fig. 1C). Administration of L-AP4 increased hippocampal neuronal survival from ~45 to a maximum of 75% during anoxia and from ~30 to 60% during NO exposure. The most significant effects occurred in the range of 250–1,000 μM (Fig. 1C).

Antagonism of the metabotropic glutamate receptor during anoxia and NO exposure is not protective

In some models of hypoxia, antagonism of metabotropic glutamate receptor function is neuroprotective. The agent L-AP3 is a partial noncompetitive antagonist of ACPD-sensitive receptors (Aramori and Nakanishi, 1992) and is an effective inhibitor of cAMP formation (Winder and Conn, 1993). Under conditions of mild hypoxic injury, L-AP3 can protect synaptic transmission in rat CA1 neurons (Opitz and Reymann, 1991). It appears that under specific environmental conditions, antagonism of the metabotropic receptor is necessary to modulate neuronal survival.

We therefore examined whether metabotropic receptor antagonism with L-AP3 during exposure to anoxia or NO could alter neuronal survival. L-AP3 was not toxic to hippocampal neurons but modestly decreased survival from ~85 to 80% at doses of 750–1,000 μM (Fig. 1D). Administration of L-AP3 1 h before anoxia or SNP administration had no significant effect on neuronal survival (Fig. 1D). Neuronal survival ranged from 16 to 30% in cultures exposed only to anoxia or treated with only SNP. In cultures pretreated with L-AP3 (10–1,000 μM) before anoxia or SNP administration, neuronal survival was not significantly altered and ranged from ~20 to 30%.

Neuroprotection by ACPD-sensitive metabotropic glutamate receptors may be mediated through the PKC pathway

We next examined whether protection through metabotropic glutamate receptor activation during anoxia or NO exposure was dependent on the PKC pathway. Initially, we examined the effects of inhibition of PKC activity without metabotropic glutamate receptor manipulation during anoxia and NO exposure. A chronic 24-h treatment with PMA (1 μM) down-regulates PKC activity but has minimal effect on the cAMP-dependent kinase activity (Matthies et al., 1987). We also inhibited the activity of PKC with the agent H-7 (10 μM) (K_i = 6.0 μM) (Hidaka et al., 1984). Down-regulation of PKC activity with PMA (1 μM) or inhibition of PKC activity with H-7 increased neuronal survival from ~25% in cultures exposed to only anoxia or NO to ~65% in cultures in which PKC had been down-regulated or inhibited (Fig. 2A and B). Administration of PMA (1 μM) alone for 24 h was not toxic to hippocampal neurons (data not shown). A 24-h pretreatment with PDD (1 μM), an inactive phorbol ester derivative, before anoxia or NO exposure did not alter neuronal survival in cultures exposed to only anoxia or NO (Fig. 2A and B), suggesting that down-regulation of PKC

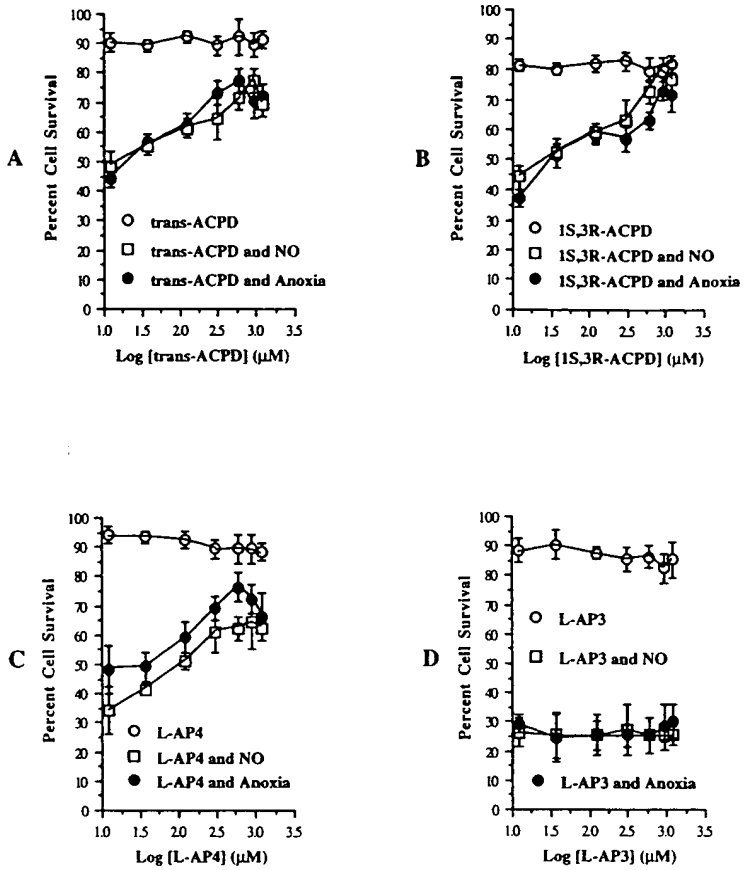

FIG. 1. Metabotropic glutamate receptor activation is neuroprotective during anoxia and NO exposure. Hippocampal cultures were pretreated with increasing concentrations of (**A**) *trans*-ACPD, (**B**) 1*S*,3*R*-ACPD, (**C**) L-AP4, and (**D**) L-AP3 1 h before an 8-h period of anoxia (●) or a 5-min exposure of SNP (300 μ*M*; □) or in the absence of anoxia or SNP (○). Neuronal survival was based on the percentage of the total number of neurons (viable plus nonviable) and determined by trypan blue exclusion 24 h following exposure to anoxia or SNP or 25 h following exposure to the individual agents in the absence of anoxia or SNP. Data are mean ± SEM (bars) values [n = 8 determinations (culture plates)] from six separate experimental preparations.

is responsible for the resistance of these cells to anoxia and NO.

PKC inhibition/1S,3R-ACPD. To determine whether PKC activity modulated the protective ability of metabotropic glutamate receptor activation during exposure to anoxia or SNP, we inhibited the activity of PKC with PMA (1 μ*M*) or H-7 during administration of the ligand 1*S*,3*R*-ACPD or L-AP4. Metabotropic receptor activation with 1*S*,3*R*-ACPD (750 μ*M*) in conjunction with inhibition of PKC activity did not significantly alter neuronal survival during anoxia and NO exposure when compared with neuronal survival following treatment with 1*S*,3*R*-ACPD in the absence of PKC activity inhibition. For example, combined inhibition of PKC activity with PMA (1 μ*M*) or H-7 and pretreatment with 1*S*,3*R*-ACPD (750 μ*M*) increased neuronal survival to ~80% during anoxia and to a maximum of 81% during NO exposure (Fig. 2A). This degree of protection is better than the neuronal survival observed with the individual inhibition of PKC activity (~65%) but does not differ significantly from the neuronal survival observed with the individual treatment of 1*S*,3*R*-ACPD (750 μ*M*) during NO administration (78%) and during anoxia (71%) (Fig. 2A).

PKC inhibition/L-AP4. In contrast to the results observed with combined inhibition of PKC activity and treatment with 1*S*,3*R*-ACPD, combined inhibition of PKC activity and administration of L-AP4 (750 μ*M*) significantly improved survival when compared with the individual treatment with PMA, H-7, or L-AP4 (Fig. 2B). During anoxia, independent inhibition of PKC activity increased neuronal survival from 26 ± 3 to 63 ± 4% with PMA (1 μ*M*) and to 62 ± 3% with H-7. Treatment with L-AP4 alone during anoxia increased neuronal survival to 70 ± 5%. Neuronal survival was significantly improved to a maximum of 87% with combined inhibition of PKC activity and L-AP4 administration (Fig. 2B). This degree of improved survival with combined inhibition of PKC activity and metabotropic receptor activation by L-AP4 was paralleled during exposure to NO (Fig. 2B).

Metabotropic glutamate receptor activation is protective during combined PKC activation and exposure to anoxia or NO

We have previously demonstrated that activation of PKC during NO exposure is detrimental to hippocampal neurons in culture (Maiese et al., 1993*b*,*c*). Yet, peptide growth factors, such as basic fibroblast growth factor and epidermal growth factor, appear to require a threshold level of PKC activation to exert their pro-

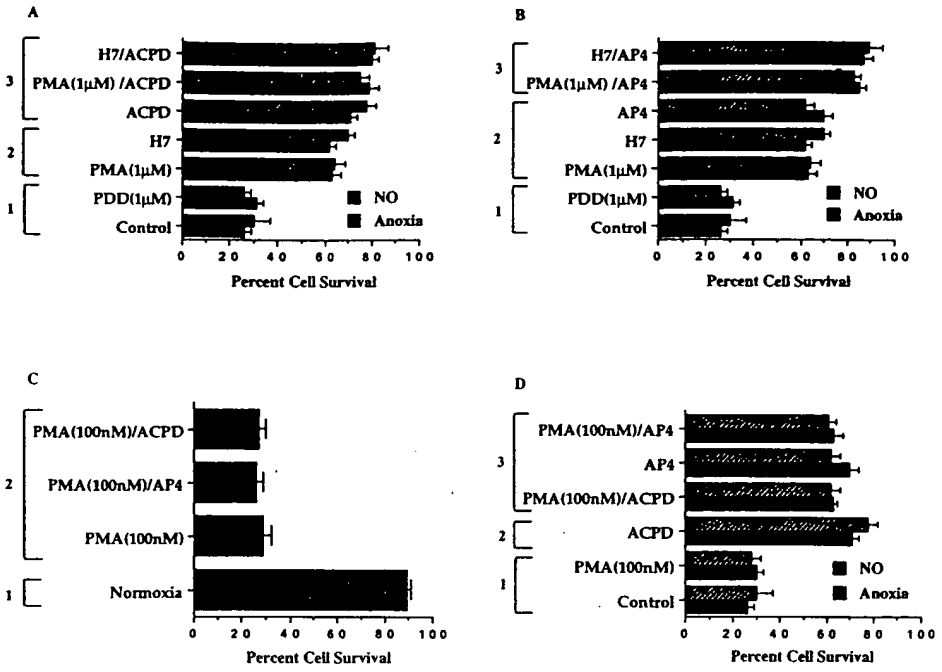

FIG. 2. Neuroprotection by ACPD-sensitive metabotropic glutamate receptors is mediated via inhibition of PKC activity. **A** and **B:** Hippocampal cultures received a 24-h pretreatment with PMA (1 μM) or PDD (1 μM) or a 1-h pretreatment with H-7 (10 μM) before an 8-h period of anoxia or a 5-min exposure of SNP (300 μM). Control cultures were exposed to only anoxia or SNP (300 μM). 1*S*,3*R*-ACPD (750 μM) and L-AP4 (750 μM) were administered 1 h before anoxia or SNP administration. Results of neuronal survival among groups 1, 2, and 3 during anoxia or NO generation are statistically different with $p < 0.001$, by ANOVA. In A, ACPD (Anoxia) is statistically different from H7/ACPD (Anoxia) using unpaired Student's *t* test ($p < 0.01$). **C** and **D:** Hippocampal cultures received a 1-h pretreatment with PMA (100 nM) before normoxia (C), anoxia (D), or SNP (300 μM) administration (D). 1*S*,3*R*-ACPD and L-AP4 were administered during normoxia or 1 h before anoxia or SNP administration. In C, results of neuronal survival between groups 1 and 2 during normoxia are statistically different with $p < 0.001$, by ANOVA. In D, results of neuronal survival among groups 1, 2, and 3 during anoxia or NO generation are statistically different with $p < 0.001$, by ANOVA. Neuronal survival was based on the percentage of the total number of neurons (viable plus nonviable) and determined by trypan blue exclusion 24 h following exposure to PMA (100 nM), anoxia, or SNP. Data are mean ± SEM (bars) values [n = 8 determinations (culture plates)] from six separate experimental preparations.

tective effects (Maiese, 1994; Maiese and Boccone, 1995). We therefore examined whether activation of PKC could alter the neuroprotective effects of metabotropic receptor activation during anoxia and NO exposure. We activated PKC by acutely pretreating the cultures with PMA (100 nM) 1 h before anoxia or SNP administration. A 100 nM dose of PMA has been shown to be sufficient to activate PKC (Lai and El-Fakahany, 1987).

Normoxia. During normoxia, PMA (100 nM) decreased neuronal survival from 89 ± 2 in untreated normoxic cultures to 29 ± 3% (Fig. 2C). Administration of 1*S*,3*R*-ACPD or L-AP4 during activation of PKC with PMA (100 nM) did not significantly alter survival during normoxia when compared with individual treatment with PMA (100 nM) during normoxia (Fig. 2C). This suggests that, at least during normoxia, the toxic events of PKC activation occur at a point distal to the activation of metabotropic glutamate receptors.

Anoxia. Results obtained with acute activation of PKC and metabotropic receptor activation during anoxia or NO exposure did not parallel the data obtained during activation of PKC with normoxia. During anoxia with PMA (100 nM) administration, neuronal survival did not differ significantly from cultures exposed to anoxia alone (Fig. 2D). Yet, metabotropic receptor activation with either 1*S*,3*R*-ACPD or L-AP4 in conjunction with activation of PKC during anoxia increased neuronal survival. During anoxia alone, neuronal survival was 26 ± 3%. Administration of 1*S*,3*R*-ACPD with PMA (100 nM) increased hippocampal neuronal survival to 63 ± 2% (Fig. 2D), and application of L-AP4 with PMA (100 nM) increased survival to 63 ± 4% (Fig. 2D).

NO exposure. Similar to the experiments with anoxia, metabotropic glutamate receptor activation with PKC activation during NO exposure improved neuronal survival when compared with NO exposure and PKC activation alone. Activation of the metabotropic glutamate receptor during PKC activation and SNP administration increased neuronal survival to 62 ± 4% with 1*S*,3*R*-ACPD and to 61 ± 3% with L-AP4 (Fig. 2D).

Neuronal survival during metabotropic glutamate receptor activation may be partially dependent on the PKA pathway

Because activation of PKA can increase neuronal survival, we examined whether modulation of PKA activity alters the neuroprotective effects of the metabotropic glutamate receptors during anoxia and NO toxicity.

PKA activation. We activated PKA by administering the cAMP analogue dbcAMP (1,000 μM) 1 h before anoxia or exposure to NO. Consistent with our previous work (Maiese et al., 1993*b*), PKA activation was neuroprotective. During anoxia, activation of PKA increased neuronal survival from 27 ± 3 to 63 ± 3%. To a similar degree of protection, PKA activation during SNP administration increased survival from 29 ± 3 to 61 ± 3% (Fig. 3A). Activation of PKA in conjunction with administration of 1*S*,3*R*-ACPD slightly improved survival to 76 ± 4% during anoxia and to 69 ± 3% during NO exposure. Yet, this degree of protection does not represent a significant improvement from individual treatment with 1*S*,3*R*-ACPD during anoxia (71 ± 3%) or during SNP administration (78 ± 4%; Fig. 3A). A marginal enhancement in survival, when compared with treatment with only dbcAMP, also was present with the combined administration of dbcAMP and L-AP4. Following combined PKA activation and treatment with L-AP4, neuronal survival was 67 ± 6% during anoxia and 64 ± 4% during SNP administration (Fig. 3A). This degree of improved survival parallels the results observed with administration of L-AP4 alone during anoxia (70 ± 5%) and NO exposure (62 ± 4%).

PKA inhibition. To examine the effects of inhibition of PKA activity on the protective ability of metabotropic receptor activation, we administered the compound H-89 (1 μM) (K_i = 48 nM), a selective inhibitor of PKA activation (Chijiwa et al., 1990). Inhibition of PKA activity with H-89 alone was not protective during anoxia or NO toxicity (Fig. 3B). Yet, combined administration of H-89 and 1*S*,3*R*-ACPD did protect hippocampal neurons during anoxia and NO administration and increased neuronal survival to 71 ± 6% during anoxia and to 68 ± 4% during NO exposure. In contrast, the protective ability of L-AP4 was compromised during inhibition of PKA activity. Combined metabotropic glutamate receptor activation with L-AP4 and inhibition of PKA activity with H-89 decreased survival to 53 ± 4% during anoxia and to 54 ± 3% during NO exposure (Fig. 3B).

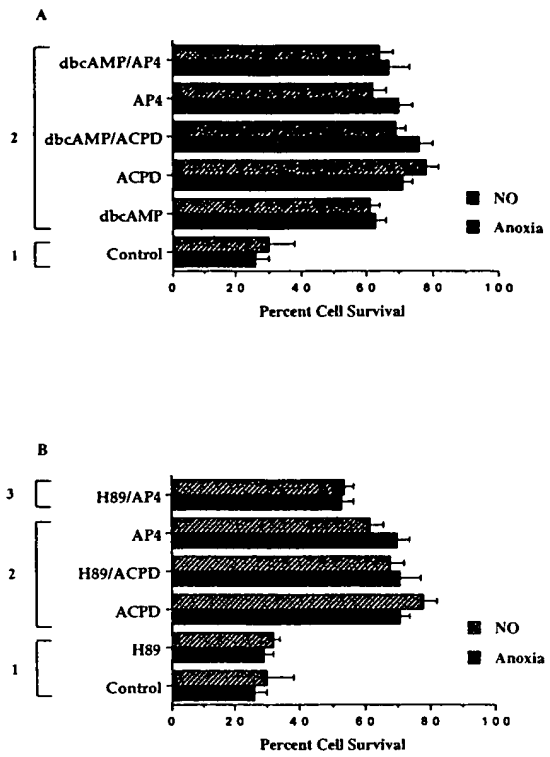

FIG. 3. Metabotropic glutamate receptor protection requires the activation of PKA. **A:** Hippocampal neuronal cultures were exposed to dbcAMP (1,000 μM) 1 h before an 8-h period of anoxia or a 5-min exposure of SNP (300 μM). Results of neuronal survival between groups 1 and 2 during anoxia or NO generation are statistically different with $p < 0.001$, by ANOVA. dbcAMP (Anoxia) is statistically different from dbcAMP/ACPD (Anoxia) using unpaired Student's *t* test ($p < 0.001$), and dbcAMP (NO) is statistically different from ACPD (NO) using unpaired Student's *t* test ($p < 0.001$). **B:** Hippocampal cultures were pretreated with H-89 (1 μM) 1 h before anoxia or exposure of SNP (300 μM). Results of neuronal survival among groups 1, 2, and 3 during anoxia or NO generation are statistically different with $p < 0.001$, by ANOVA. ACPD (NO) is statistically different from AP4 (NO) using unpaired Student's *t* test ($p < 0.001$). For both A and B, control cultures were exposed to only anoxia or SNP (300 μM). 1*S*,3*R*-ACPD (750 μM) and L-AP4 (750 μM) were administered 1 h before anoxia or SNP administration. Neuronal survival was based on the percentage of the total number of neurons (viable plus nonviable) and determined by trypan blue exclusion 24 h following exposure to anoxia or SNP. Data are mean ± SEM (bars) values [n = 8 determinations (culture plates)] from six separate experimental preparations.

Metabotropic glutamate receptor activation following an NO insult is neuroprotective

To determine whether metabotropic glutamate receptor activation is neuroprotective following exposure to NO, we administered 1*S*,3*R*-ACPD (750 μM) and L-AP4 (750 μM) at 1, 2, 4, 6, 12, and 24 h following the onset of SNP (300 μM) administration. During 1*S*,3*R*-ACPD treatment, neuronal survival increased from ~30% during NO exposure alone to 67 ± 5% with 1*S*,3*R*-ACPD administered at the time of SNP application (time = 0 h) and to 73 ± 2% with application of 1*S*,3*R*-ACPD 1 h following exposure to NO (Fig. 4). A significant degree of protection remained with 1*S*,3*R*-ACPD administered 2 (59 ± 3% survival) and 4 h (51 ± 4% survival) after SNP application. A modest degree of protection was present at 6 h posttreatment with 1*S*,3*R*-ACPD, with a survival of 41 ± 3%, but no significant improvement in neuronal survival was evident at the posttreatment periods of 12 and 24 h following NO exposure (Fig. 4).

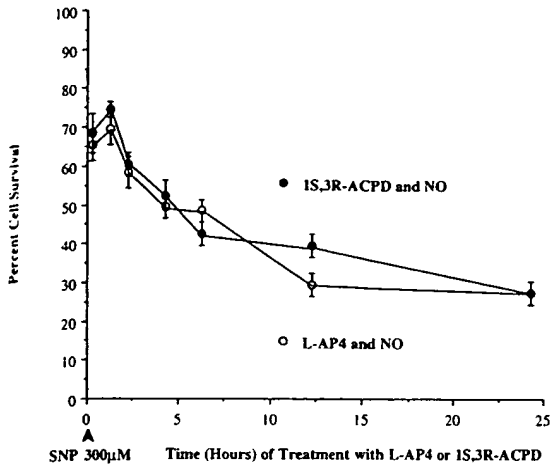

FIG. 4. A "window of opportunity" exists during metabotropic glutamate receptor activation. Hippocampal cultures received 1*S*,3*R*-ACPD (750 μM) and L-AP4 (750 μM) at 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h following the onset of SNP (300 μM) administration. Neuronal survival was based on the percentage of the total number of neurons (viable plus nonviable) and determined by the trypan blue exclusion method. In the absence of SNP, the percentage of viable cells was 90 ± 5%, whereas SNP reduced viability to 26 ± 4%. Data are mean ± SEM (bars) values [n = 8 determinations (culture plates)] from six separate experiments.

Analogous to the posttreatment protection conferred by 1*S*,3*R*-ACPD, administration of L-AP4 at the time of SNP administration or at 1 and 2 h following NO exposure was neuroprotective (Fig. 4). Neuronal survival increased from 26 ± 4% during SNP treatment alone to 64 ± 4% with administration of L-AP4 at the time of NO exposure and to 68 ± 4% with L-AP4 given 1 h after SNP treatment. A significant degree of protection remained with the posttreatment times of 2 (57 ± 4%), 4 (48 ± 3%), and 6 h (47 ± 3%). No significant protection against neuronal degeneration was present with the posttreatment times of 12 and 24 h. As with 1*S*,3*R*-ACPD treatment, maximal protection during this experimental protocol was present with the 1-h posttreatment period (Fig. 4).

DISCUSSION

CNS metabotropic glutamate receptors function differently from ionotropic glutamate receptors. Ionotropic receptors form ligand complexes that regulate neuronal ionic fluxes. In contrast, metabotropic glutamate receptors are coupled to second messenger systems and function through GTP-binding proteins. Thus, metabotropic glutamate receptors and ionotropic receptors may control anoxic- or NO-induced injury through different pathways. We demonstrate that *trans*-ACPD, 1*S*,3*R*-ACPD, and L-AP4 are protective during anoxia and NO exposure.

Metabotropic glutamate receptor neuroprotection during ischemic insults is postulated to be partially mediated by protein kinases, such as PKC (see Fig. 5). PKC has been closely linked with ischemic neuronal death. Although some groups report a decrease in PKC activity 15 min following transient cerebral ischemia (Busto et al., 1994), several investigators have demonstrated an early increase in PKC activity following cerebral ischemia. The study of PKC activity is clearly dependent on several factors, which include the experimental model used, the temporal profile examined, and the specific PKC isozymes evaluated. During cerebral ischemia, PKC is translocated to cell membranes (Cardell et al., 1990), and increased levels are present in the hippocampal CA1 region (Hara et al., 1990). Increased activity of PKC occurs during global ischemia (Louis et al., 1988; Lu et al., 1993), and inhibition of PKC activation can reduce brain edema that may contribute to infarct size in the rat model (Joó et al., 1989). PKC is one of the regulatory mechanisms for NOS (Bredt et al., 1992), and inhibition of PKC activity has been shown to be neuroprotective during glutamate toxicity (Favaron et al., 1988), anoxia, and NO toxicity (Maiese et al., 1993*b*,*c*; Maiese and Boccone, 1995).

Our present results demonstrate that *activation* of PKC is detrimental to hippocampal neurons during both normoxic and anoxic environments. Although metabotropic glutamate receptor agonists do not in-

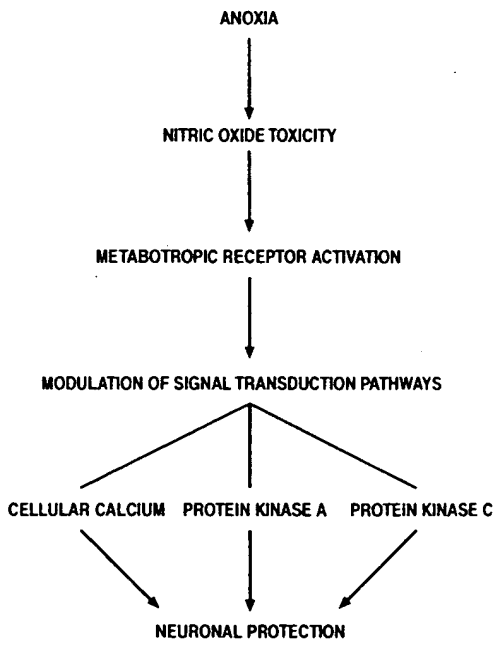

FIG. 5. Postulated mechanism of neuroprotection by metabotropic glutamate receptor activation during anoxia or NO toxicity. The present studies demonstrate that activation of metabotropic glutamate receptors is protective during anoxia or NO exposure. Our results suggest that neuronal protection via metabotropic receptor activation may be dependent on the signal transduction pathways of PKC, PKA, and cellular calcium. Neuronal survival following anoxic or NO injury may ultimately require the inhibition of PKC activity, the activation of PKA, and the maintenance of cellular calcium homeostasis.

crease neuronal survival during PKC activation in a normoxic environment, the ligands 1*S*,3*R*-ACPD and L-AP4 are protective during combined PKC activation and exposure to anoxia or NO. These data suggest that during anoxia or exposure to NO, activation of PKC results in neuronal degeneration by a mechanism that is different from the one that occurs during normoxia. It is conceivable that the toxicity of PKC activation during anoxia or NO exposure requires several intermediate steps that may be "controlled" by metabotropic glutamate receptor activation ultimately to limit neuronal cell death.

Our work suggests that the modulation of specific signal transduction pathways by metabotropic glutamate receptors may differ between normoxic and anoxic conditions. Although some metabotropic agonists can increase the activity of PKC in normoxic experimental systems (Manzoni et al., 1990), our results illustrate that neuroprotection during anoxia and NO exposure by metabotropic glutamate receptor activation may require the *inhibition* of PKC activity. This idea is supported by two observations. First, inhibition of PKC activity did not compromise neuronal protection by 1*S*,3*R*-ACPD or L-AP4 during anoxia or NO exposure. Second, protection by 1*S*,3*R*-ACPD during anoxia or NO exposure was not significantly enhanced by inhibition of PKC activity, suggesting that neuronal survival mediated by ACPD-sensitive metabotropic glutamate receptors may be, at least in part, dependent on the inhibition of PKC activity. As combined treatment with L-AP4 and inhibition of PKC activity significantly improved neuronal survival over treatment with L-AP4 alone, these results suggest that L-AP4 metabotropic receptor subtypes, such as mGluR4, may increase neuronal survival during anoxia and NO toxicity by mechanisms that are both dependent and independent of PKC modulation. Our studies demonstrate that the mechanism of protection by metabotropic receptor agonists during normoxia and during anoxia or NO exposure differs, suggesting that *inhibition* rather than activation of PKC is the protective mechanism used by the metabotropic receptor agonists.

In addition to the regulation of PKC activity, protection conferred by metabotropic receptor activation also may function via the PKA pathway (see Fig. 5). The metabotropic receptors mGluR1 and mGluR4 receptors modulate cAMP levels (Winder and Conn, 1993). Increased activity of PKA can increase survival of cells during serum deprivation (Tamm and Kikuchi, 1991) and is neuroprotective during NO exposure (Maiese et al., 1993*b*). Metabotropic receptor agonists, such as *trans*-ACPD and 1*S*,3*R*-ACPD, increase basal cAMP accumulation in mGluR1-expressing cells (Aramori and Nakanishi, 1992) and in neuronal cells (Winder et al., 1993), which leads to the activation of PKA. Our results suggest that part of the protective effects conferred by metabotropic glutamate receptor activation is mediated through the increased activity of PKA. The degree of protection by either 1*S*,3*R*-ACPD or L-AP4 during anoxia or NO exposure was not significantly enhanced by coapplication of dbcAMP, suggesting that survival mediated by metabotropic glutamate receptor activation may proceed along a common pathway involving PKA. In addition, inhibition of PKA activity slightly diminished the protective ability of the metabotropic glutamate agonist L-AP4. It appears that protection by L-AP4 and the mGluR4 receptor subtype during anoxia or NO exposure is at least partially dependent on the activation of PKA. In contrast, protection by 1*S*,3*R*-ACPD was not significantly altered during inhibition of PKA activity. These results may suggest that other signal transduction pathways, such as PKC or the mobilization of intracellular calcium, may be more responsible for the protective effects observed with 1*S*,3*R*-ACPD treatment. Future studies that directly assay PKA activity and PKC activity may provide further insight into these signal transduction pathways that are responsible for the protective role of the metabotropic glutamate receptors.

It is possible that in addition to the modulation of protein kinase activity, metabotropic glutamate receptors also may mediate neuronal protection through the regulation of cellular calcium influx (see Fig. 5). Cellular calcium influx can lead to the activation of PKC, which contributes to both glutamate-induced cell death (Favaron et al., 1988) and NO toxicity (Maiese et al., 1993*b*,*c*; Maiese and Boccone, 1995). The metabotropic glutamate agonists *trans*-ACPD and 1*S*,3*R*-ACPD can suppress the activity of high-voltage calcium channels in dissociated CA3 rat hippocampal neurons (Swartz and Bean, 1992). The agonist L-AP4 also has been shown to inhibit independently cellular calcium influx (Trombley and Westbrook, 1992). Thus, it is conceivable that metabotropic receptor activation prevents neurodegeneration by maintaining either extracellular or intracellular calcium homeostasis and inhibiting the calcium-mediated generation of NO (Maiese et al., 1994*b*). Further work along this avenue will be necessary to confirm the role of calcium in metabotropic glutamate receptor-mediated neuroprotection.

In contrast to the protective effects observed with the metabotropic glutamate receptor agonists, antagonists of the metabotropic receptors, such as L-AP3, do not appear to increase hippocampal neuronal survival during anoxia and NO exposure. In our studies, treatment with L-AP3 was ineffective in preventing neuronal cell death during anoxia and NO exposure. The agent L-AP3 blocks the activation of cAMP by metabotropic glutamate receptor agonists (Winder and Conn, 1993). It is not known whether L-AP3 alters the function of metabotropic glutamate receptors that stimulate cAMP activity or whether L-AP3 directly decreases cAMP activity. Given the protective nature of PKA activation during NO toxicity (Maiese et al., 1993*b*), it is possible that the inability of L-AP3 to confer protection during ischemic insults and NO exposure is a result of inhibition of PKA activity, either at the level

of the metabotropic receptor or through direct inhibition of cAMP activity.

Our work suggests that a "window of opportunity" exists for protection against NO toxicity. Activation of metabotropic receptors during a period of ~6 h following NO exposure increased neuronal survival. It appears that metabotropic receptor activation can prevent and reverse a previously sustained cellular insult. This suggests that the mechanisms of cellular death are a more dynamic process that can be altered rather than a fixed cellular event that ultimately results in neuronal cell death. These results are obviously very attractive when extrapolated to the clinical treatment of neurodegenerative disease. Reduction in neuronal cell death and clinical disability may be achieved by regulation of metabotropic glutamate receptor activity following the onset of the initial insult.

We have previously shown that the signal transduction pathways of PKC and PKA can mediate both the protective effects of peptide growth factors and the detrimental effects of NO toxicity (Maiese et al., 1993*b*,*c*; Maiese and Boccone, 1995). The present study further dissects the signal transduction pathways that regulate neuronal survival. Modulation of metabotropic glutamate receptor activity can prevent neurodegeneration and reverse a previously sustained insult following anoxia or NO exposure. The cellular mechanisms that mediate the protective effects of the metabotropic glutamate receptors are dependent, at least in part, on the pathways of PKC and PKA, suggesting that modulation of these cellular signal transduction systems may provide a future therapeutic target for neurodegenerative disease.

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The Inhibitory mGluR Agonist, s-4-carboxy-3-hydroxy-phenylglycine Selectively Attenuates NMDA Neurotoxicity and Oxygen–Glucose Deprivation-induced Neuronal Death

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Summary—We examined the effect of two novel phenylglycine derivative drugs on excitotoxicity in murine cortical cell cultures: s-4-carboxy-3-hydroxy-phenylglycine (4C3HPG), a selective agonist of mGluRs 2/3 and an antagonist at mGluRs 1/5, and s-3 hydroxy-phenylglycine (3HPG), an agonist of mGluRs 1/5. 4C3HPG attenuated slowly-triggered NMDA-induced excitotoxic neuronal death, as well as the death induced by combined oxygen–glucose deprivation, but did not affect slowly-triggered excitotoxicity induced by AMPA or kainate. As expected, 4C3HPG also reduced NMDA-induced increases in cAMP in near-pure neuronal cultures, and the protective effect of 4C3HPG on NMDA toxicity could be reversed by adding 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic-monophosphate (CPT cAMP) to the exposure medium. In contrast, 3HPG did not did not have any protective effects in these paradigms; in fact, slowly-triggered NMDA-induced excitotoxicity and the neuronal cell death induced by oxygen–glucose deprivation were potentiated. These results are consistent with the idea that the "inhibitory" mGluRs 2/3 exert a negative modulatory action on NMDA receptor-mediated excitotoxicity via reduction in neuronal cAMP levels.

Keywords—Excitotoxicity, metabotropic glutamate receptor, cortical neurons, oxygen–glucose deprivation, NMDA, phenylglycine derivatives.

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors which participate broadly in glutamate neurotransmission (Schoepp and Conn, 1993). Eight major subtypes have been cloned, classifiable by sequence and action into three groups: mGluR 1 and 5, which are positively coupled to phosphoinositol hydrolysis; mGluR 2 and 3, which are negatively coupled to adenylate cyclase; and mGluR 4, 6, 7 and 8, which are also negatively linked to adenylate cyclase, but show a different agonist preference than the mGluR 2/3 subtypes (Pin and Duvoisin, 1995).

Substantial evidence from many laboratories indicates that central neurotoxicity mediated by neurotransmitter glutamate—excitotoxicity—can contribute to several forms of central nervous system damage, including that induced by hypoglycemia, hypoxia–ischemia and trauma (Meldrum, 1985; Rothman and Olney, 1987; Choi, 1992). This excitotoxic neuronal injury is mediated by activation of ion channel-linked glutamate receptors, especially *N*-methyl-D-aspartate (NMDA) receptors, and subsequent excessive Ca^{2+} influx. It is expected that mGluRs will be activated concurrently with activation of ion channel-linked glutamate receptors, but the consequences of mGluR receptor activation on excitotoxic injury remains currently unclear.

Koh *et al.* (1991) reported that activation of mGluRs with the broad spectrum agonist, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*ACPD) attenuated rapidly-triggered NMDA neurotoxicity in cortical neurons. Siliprandi *et al.* (1992) reported that intraoccular injection of 1S,3R ACPD reduced NMDA-induced retinal damage. In addition, Sheardown (1992) found that epileptiform activity in rat neocortical slices was attenuated by 1S,3R ACPD. However, in contrast, Aleppo *et al.* (1992) found that *t*ACPD potentiated NMDA-induced cell death in cerebellar granule cell culture, and Saccaan and Schoepp (1992) found that intrahippocampal injection of 1S,3R ACPD resulted in limbic seizures and hippocampal cell loss.

These opposing effects may reflect the utilization of a broad spectrum mGluR agonist, activating multiple

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mGluR subtypes and inducing a multiplicity of consequences, some protective and some injurious. In particular, *t*ACPD activates both the "inhibitory" mGluR subtypes, mGluRs 2/3, which inhibit cAMP formation and overall tend to suppress glutamate neurotransmission (Nakanishi, 1992), and the more excitatory mGluR subtypes, mGluRs 1/5, which mobilize intracellular Ca^{2+} and hence might be expected to increase excitotoxic injury. Key support for this idea was reported by Bruno *et al.* (1994), who found that (2s,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), a preferential agonist at the mGluR 2/3 group, exerted a protective effect against excitotoxicity in cortical cell cultures.

Recently, substances structurally related to phenylglycine were discovered to have selective agonist actions on different mGluRs subtypes (Watkins and Collingridge, 1994). The purpose of the present experiments was to test the above hypothesis of mGluR 2/3 neuroprotection against excitotoxicity, using two especially interesting phenylglycine derivatives: s-4-carboxy-3-hydroxy-phenylglycine (4C3HPG), a selective agonist of mGluRs 2/3 and an antagonist at mGluRs 1/5; and s-3 hydroxy-phenylglycine (3HPG), an agonist of mGluRs 1/5.

An abstract has appeared (Buisson *et al.*, 1994).

METHODS

Cell culture

Culture medium was obtained from GIBCO (Grand Island, NY) as 10 × concentrated stock lacking bicarbonate and glutamine; serum was from Hyclone laboratories Inc. (Logan, UT). All other chemicals were purchased from Sigma (St Louis, MO, U.S.A.).

Mixed cortical cell cultures containing both neuronal and glial elements, were prepared from fetal mice at 15–16 days gestation (Rose *et al.*, 1993). Cerebral cortices were dissected and incubated for 20–30 min in 0.025% of trypsin in media stock (MS, modified Eagle's minimal essential medium with 2 mM glutamine and 25 mM glucose) and transferred to MS supplemented with 5% fetal bovine and 5% horse serum for trituration. Dissociated cells were plated at a density of about 3×10^5 cells per well on an established bed of glia in MS supplemented with 10% fetal bovine serum and 10% horse serum. Glial cell cultures from cortex were prepared similarly from 1-to 3-day-old postnatal pups, using plating medium supplemented with epidermal growth factor (10 ng/ml) (Rose *et al.*, 1993).

Pure neuronal cell cultures containing < 1% of astrocytes were prepared as previously detailed (Hewett *et al.*, 1994). In brief, dissociated cortical cells were plated in multiwell vessels that had been previously coated with poly-D-lysine and laminin, using conditioned medium from astrocytic cultures with 3 μM cytosine arabinoside (procedure developed by L. Dugan, personal communication). There was no further exchange of the media. Experiments were performed on cultures after 12–13 days *in vitro*.

All cultures were kept at 37°C in a humidified 5% CO_2-containing atmosphere. After 3–7 days *in vitro* glial cell division was halted by exposure to 10 μM cytosine arabinoside. Cells were subsequently shifted into maintenance medium which was identical to the plating medium, but lacking fetal bovine serum. The medium was changed twice weekly. Experiments were performed on cortical cultures after 14–15 days *in vitro*.

Excitotoxicity

Slowly-triggered excitotoxicity was carried out at 37°C and was induced by a 24 hr exposure to low concentration of NMDA (15 μM), AMPA (7.5 μM) or kainate (35 μM) in MS supplemented with glycine. MK-801 (10 μM) was always added concurrently with AMPA or kainate to block secondary NMDA receptor activation.

Rapidly-triggered excitotoxicity was induced by 5 min exposure to high concentrations (200 μM) of NMDA, carried out at room temperature in a HEPES buffer solution (HBSS) containing (in mM): NaCl, 120; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 20; glucose, 15; glycine, 0.01 (pH 7.4). The exposure solution was then washed away, and replaced by MS supplemented with 10 μM glycine before returning cultures to the incubator for 20–24 hr.

Combine oxygen–glucose deprivation

Cultures were transferred to an anaerobic chamber (Forma Scientific) containing a gas mixture of 5% CO_2 10% H_2, 85% N_2. The culture media was replaced by thorough exchange (effective dilution < 1:1000) with deoxygenated, glucose-free Eagle's minimum essential medium (pH 7.4). Cultures were then placed in a humidified 37°C incubator within the anaerobic chamber. Oxygen–glucose deprivation was terminated by replacing the exposure medium with MS supplemented with 10 μM glycine, and cultures were returned to normoxic incubator. Neuronal cell death was assessed 24 hr later.

Measurement of cAMP formation

Pure neuronal cultures were exposed for 5 min at room temperature in a HEPES buffer solution (HBSS) to high concentrations (200 μM) of NMDA and 3-isobutyl-1-methylxanthine (500 μM), a non-specific phosphodiesterase inhibitor, in the presence of 4C3HPG or MK 801. The incubation was terminated by adding 1 ml of acetonitrile (1 M) after complete removal of the medium. Cells were harvested with a scraper, and cAMP was extracted using ion-exchange chromatography (Amprep SAX 500 mg

minicolumn, Amersham), and quantitated using a commercially-available scintillation proximity assay system (Amersham, RPA 538).

Assessment of neuronal cell death

Neuronal death was estimated by examination of the cultures under phase-contrast microscopy, and quantitated measurement of lactate dehydrogenase (LDH) release by damaged cells into the bathing medium one day following the experimentation (Koh and Choi, 1987). The LDH level corresponding to complete neuronal death (without glial death) was determined by assaying sister cultures exposed to 200 μM NMDA for 24 hr in MS supplemented with glycine. Background LDH levels were determined in sister cultures subjected to sham wash and subtracted from experimental values to yield the signal specific to experimentally-induced injury.

Data was analyzed using one-way analysis of variance (ANOVA) and Student–Neuman–Keuls' multiple comparisons test.

RESULTS

We first tested the effect of 4C3HPG on slowly-triggered forms of excitotoxic injury (Choi, 1992). When mixed neuron–glial cultures neurons were exposed to low concentration of NMDA (15 μM) for 24 hr, about 75% of the neurons were destroyed whereas the glia remained intact. Addition of 1–100 μM 4C3HPG reduced neuronal cell death in a concentration dependent fashion, with maximum effect at 50–100 μM (Fig. 1A). In contrast, neither AMPA-nor kainate-induced slowly-triggered excitotoxic neuronal cell death (conducted in the presence of 10 μM MK-801 to block secondary activation of NMDA receptors) were reduced by addition of 100 μM 4C3HPG (Fig. 1B).

We next tested the hypothesis that the protective effect of 4C3HPG on slowly-triggered NMDA toxicity was mediated mainly by its known agonist effects on mGluRs 2/3, and thus by inhibition of neuronal adenylate cyclase. Pure neuronal cultures exposed for 5 min to 200 μM NMDA showed about a 3-fold increase in cellular cAMP (Fig. 2A); this increase was completely blocked by 10 μM MK-801, and partially blocked by 1 μM 4C3HPG, the lowest concentration of 4C3HPG showing neuroprotective activity. Furthermore, addition a 500 μM concentration of the stable membrane-permeable cAMP analog, 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic-monophosphate (CPTcAMP) CPTcAMP (500 μM) was without effect on an intermediate level of slowly-triggered NMDA neurotoxicity, but reversed the neuroprotective effect of 100 μM 4C3HPG (Fig. 2B).

In contrast to the protective effect of 4C3HPG, the phenylglycine mGluR 1/5 agonist, 3HPG, at 10–200 μM increased slowly-triggered NMDA neurotoxicity in a concentration-dependent fashion (Fig. 3A). No significant effect was produced by 100 μM 3HPG on slowly-triggered AMPA-or kainate-induced neuronal

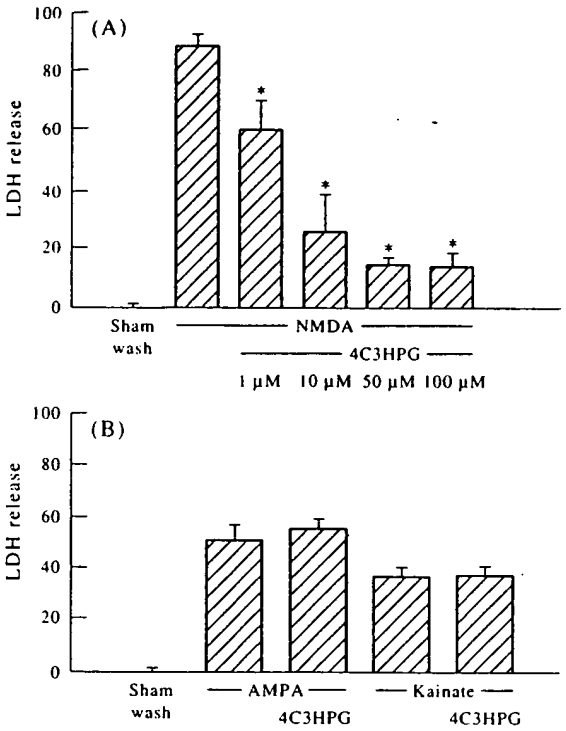

Fig. 1. 4C3HPG attenuates slowly-triggered excitotoxicity. (A) Slowly-triggered NMDA toxicity. Cultures were exposed for 24 hr to NMDA (15 μM) alone or in the presence of the indicated concentration of 4C3HPG. LDH released to the bathing medium was assessed at the end of the excitotoxin exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). * Indicates significantly different from NMDA alone by ANOVA followed by Student–Newman–Keuls' test ($P < 0.05$). (B) Slowly-triggered AMPA or kainate toxicity. Cultures were exposed for 24 hr to AMPA (7.5 μM) or kainate (35 μM) (with 10 μM MK-801 added to block secondary NMDA receptor-mediated toxicity), without or with 100 μM 4C3HPG. Medium LDH were assessed at the end of excitotoxins exposure (mean $\pm$ SEM, $n = 12$ cultures per condition).

death (Fig. 3B). Potentiation of slowly-triggered NMDA neurotoxicity was blocked by inclusion of the mGluR antagonist compound, α-methyl-4-carboxyphenyl-glycine (MCPG) (Hayashi *et al.*, 1994), at 500 μM (Fig. 3C).

4C3HP (100 μM) also reduced rapidly-triggered NMDA-induced neuronal death; 100 μM 3HPG was without significant effect (Fig. 4).

Consistent with their effects on slowly-triggered NMDA neurotoxicity, 4C3HPG and 3HPG altered neuronal cell death induced by combined oxygen–glucose deprivation (Monyer *et al.*, 1989), a paradigm which we have previously shown induces NMDA receptor-mediated neuronal injury (Goldberg and Choi, 1993). When mixed neuronal-glial cultures were exposed to oxygen–glucose deprivation for 55 min, about 80% of the neurons degenerated by the next day; glia remained intact. 1–100 μM 4C3HPG produced a concentration-dependent neuroprotective effect (Fig. 5A), and the protective effect of 100 μM 4C3HPG was blocked by

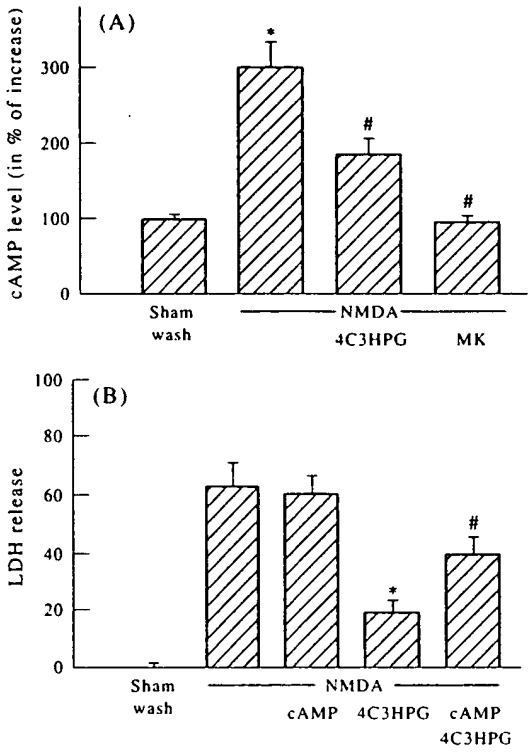

Fig. 2. Involvement of cAMP reduction in 4C3HPG neuroprotection. (A) 4C3HPG reduces NMDA-induced increases in neuronal cAMP. Pure neuronal cultures were exposed for 5 min to NMDA (200 μM) with IBMX (500 μM), alone or in the presence of 4C3HPG (1 μM) or MK 801 (10 μM). Values are mean $\pm$ SEM ($n = 4$ cultures per condition). * Indicates significantly different from sham wash at $P < 0.05$; # indicates significantly different from NMDA alone at $P < 0.05$ (ANOVA followed by Student–Newman–Keuls' test). (B) Addition of CPTcAMP reduces 4C3HPG neuroprotection. Cultures were exposed for 24 hr to NMDA (15 μM) alone, in the presence of 4C3HPG (100 μM), or in the presence of 4C3HPG + CPTcAMP (500 μM). Medium LDH were assessed at the end of NMDA exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). * Indicates significantly different from NMDA alone at $P < 0.05$; # indicates significantly different from NMDA + 4C3HPG at $P < 0.05$ (ANOVA followed by Student–Newman–Keuls' test).

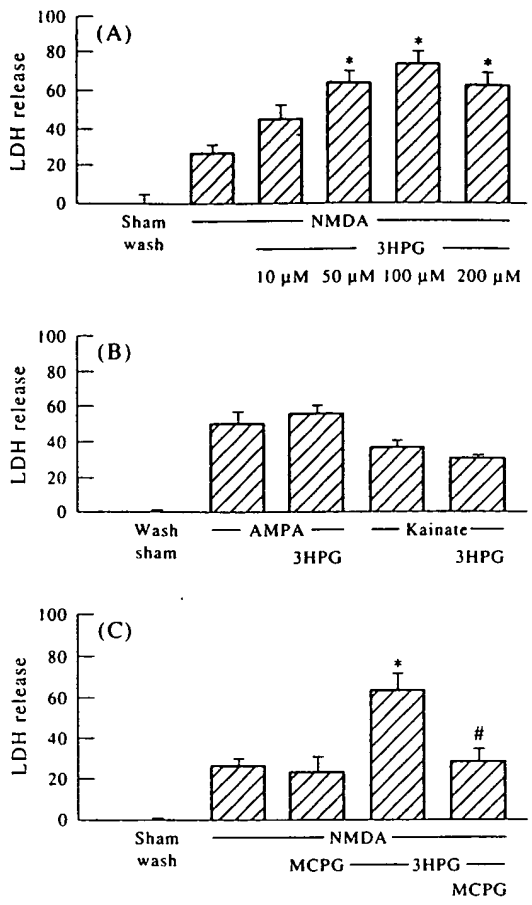

Fig. 3. 3HPG does not block slowly-triggered excitotoxicity. (A) Slowly-triggered NMDA toxicity. Cultures were exposed for 24 hr to NMDA (10 μM) alone or in the presence of the indicated concentration of 3HPG. LDH released to the bathing medium was assessed at the end of the excitotoxin exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). * Indicates significantly different from NMDA alone by ANOVA followed by Student–Newman–Keuls' test ($P < 0.05$). (B) Slowly-triggered AMPA or kainate toxicity. Cultures were exposed for 24 hr to AMPA (7.5 μM) or kainate (35 μM) (with 10 μM MK-801 added to block secondary NMDA receptor-mediated toxicity), without or with 100 μM 3HPG. Medium LDH were assessed at the end of excitotoxins exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). (C) Slowly-triggered NMDA toxicity. Cultures were exposed for 24 hr to NMDA (10 μM) in the presence of 3HPG (100 μM), or in the presence of 3HPG + MCPG (500 μM). LDH released to the bathing medium was assessed at the end of the excitotoxin exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). * Indicates significantly different from NMDA alone at $P < 0.05$; # indicates significantly different from NMDA + 3HPG at $P < 0.05$ (ANOVA followed by Student–Newman–Keuls' test).

coapplication of 500 μM CPT cAMP (Fig. 5B). In contrast, the low level of neuronal death (about 20% of the neuronal population) induced by 45 min exposure to oxygen–glucose deprivation was increased by 10–200 μM 3HPG (Fig. 5C).

DISCUSSION

The observation that 4C3HPG was highly protective against both slowly-triggered and rapidly-triggered NMDA-induced neurotoxicity is consistent with prior observations of Koh *et al.* (1991), Bruno *et al.* (1994) and ourselves (Buisson *et al.*, 1994; Buisson and Choi, 1994), using other mGluR agonist drugs. Four additional new findings are presented here: (1) evidence that the protective effect of 4C3HPG is mediated, at least in part, by blocking NMDA-induced increases in neuronal cAMP levels, presumably by activating mGluRs 2/3; (2) evidence that the anti-excitotoxic effect of 4C3HPG is selective for NMDA vs AMPA/kainate receptor-mediated injury; (3) evidence suggesting that activation of the "excitatory"

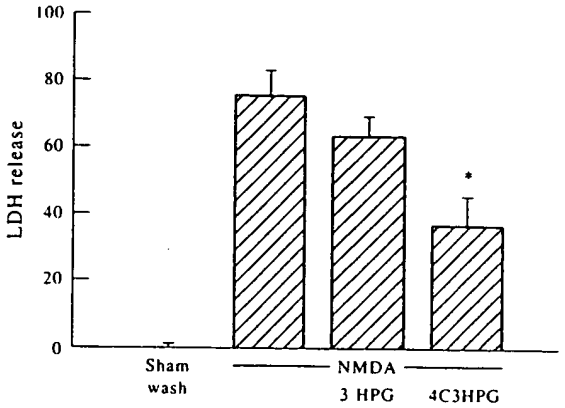

Fig. 4. 4C3HPG attenuates rapidly-triggered excitotoxicity Rapidly-triggered NMDA toxicity. Cultures were exposed for 5 min to NMDA (200 μM) alone or in the presence of 100 μM of 4C3HPG or 100 μM of 3HPG. LDH released to the bathing medium was assessed at the end of the excitotoxin exposure (mean $\pm$ SEM, $n = 12$ cultures per condition). * Indicates significantly different from NMDA alone by ANOVA followed by Student–Newman–Keuls' test ($P < 0.05$).

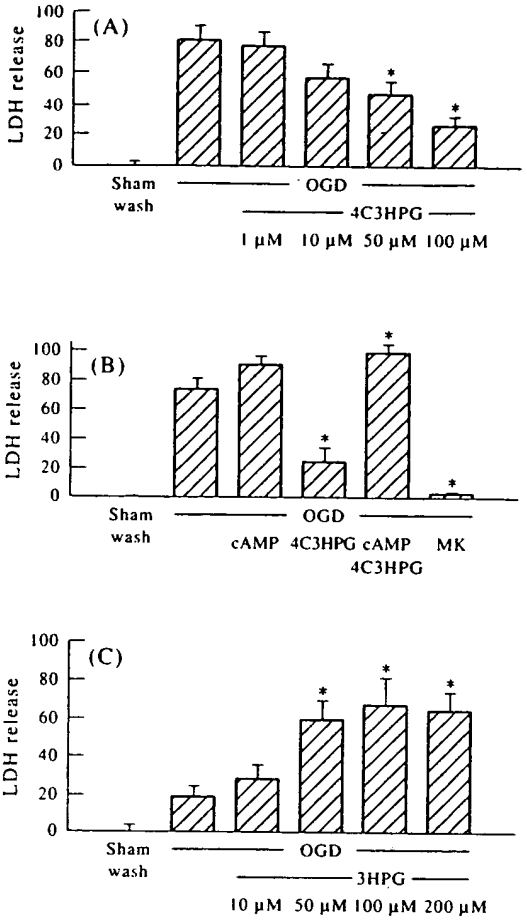

Fig. 5. 4C3HPG and 3HPG modulate neuronal death induced by oxygen–glucose deprivation. (A) Increasing concentration of 4C3HPG reduce oxygen–glucose deprivation-mediated neuronal injury. Cultures were exposed to oxygen–glucose deprivation for 55 min alone or in the presence of the indicated concentration of 4C3HPG. LDH release to the bathing medium were assessed 20–24 hr later; mean $\pm$ SEM ($n = 12$ cultures per condition) are shown. * Indicates significantly different from OGD treatment alone by one-way ANOVA followed by Student–Newman–Keuls' test for multiple comparisons ($P < 0.05$). (B) CPTcAMP reverses 4C3HPG neuroprotection against oxygen–glucose deprivation-mediated neuronal injury. Cultures were exposed to oxygen–glucose deprivation for 55 min alone, in the presence of 4C3HPG (100 μM), or in the presence of 4C3HPG + CPTcAMP (500 μM). LDH release to the bathing medium were assessed 20–24 hr later; mean $\pm$ SEM ($n = 12$ cultures per condition) are shown. * Indicates significantly different from OGD treatment alone by one-way ANOVA followed by Student–Newman–Keuls' test for multiple comparisons ($P < 0.05$); # indicates significantly different from OGD + 4C3HPG by one-way ANOVA followed by Student–Newman–Keuls' test for multiple comparisons ($P < 0.05$). (C) Increasing concentration of 3HPG-potentiated oxygen–glucose deprivation-mediated neuronal injury. Cultures were exposed to combined oxygen–glucose deprivation for 45 min alone or in the presence of the indicated concentration of 3HPG. LDH release to the bathing medium were assessed 20–24 hr later; mean $\pm$ SEM ($n = 12$ cultures per condition) are shown. * Indicates significantly different from OGD treatment alone by one-way ANOVA followed by Student–Newman–Keuls' test for multiple comparisons ($P < 0.05$).

mGluRs 1/5 by 3HPG is not neuroprotective, and perhaps injury-potentiating under some conditions; and (4) demonstration that the neuroprotective effect of 4C3HPG extends to the injury induced by combined oxygen–glucose deprivation.

The idea that NMDA receptor-mediated toxicity may be mediated in part by an evoked increase in neuronal cAMP levels is intriguing. Our observation that NMDA exposure can rapidly induce a large increase in neuronal cAMP levels is consistent with previous reports suggesting that, in hippocamppal slices, NMDA receptor activation produces an increase in cAMP level through a Ca^{2+}/calmodulin-mediated stimulation of adenylate cyclase (Chetkovich and Sweatt, 1993). Further study will be needed to understand how increased neuronal cAMP may participate in NMDA neurotoxicity. One possibility would be the ability of cAMP to increase the open time of high-threshold (Chetkovich *et al.*, 1991; Goulding *et al.*, 1994). Of note, mGluR 2/3 activation by low concentrations of (2s,1's,2's)-2-(carboxycyclopropyl)glycine (L-CCG-1) reduced L-type Ca^{2+} currents in cerebellar granule cells, although those authors concluded that the effect was not mediated by reduction of cellular cAMP levels (Chavis *et al.*, 1994). Another effect of mGluR activation (generally revealed with *t*ACPD) that may have anti-excitotoxic properties is the supression of presynaptic glutamate release (Nakanishi, 1994).

The present finding that the neuroprotective effect of 4C3HPG is selective for NMDA toxicity vs AMPA or kainate toxicity fits with our observations with DCG-IV (unpublished results), but contrasts with the broad anti-excitotoxic action observed by Bruno *et al.* (1994) with DCG-IV in cortical cell cultures. We favor the idea that this selectivity may prove to be an important clue in

delineating the mechanisms by which mGluR 2/3 can attenuate NMDA receptor-mediated toxicity.

The observation that 3HPG, presumably activating the "excitatory" mGluRs 1/5, enhanced the neuronal death induced by slowly-triggered NMDA toxicity or oxygen–glucose deprivation provides a plausible explanation for the injury potentiating effects of the broad spectrum mGluR agonist, *t*ACPD, in some systems (see above). The ability of mGluR 1/5 activation to release Ca^{2+} from intracellular stores, or to potentiate current through glutamate receptor-gated channels (Aniksztein *et al.*, 1991), may underlie this injury enhancement.

The protective effect of 4C3HPG against oxygen–glucose deprivation-induced injury documented here is consistent with prior studies linking energy depleting insults to NMDA receptor-mediated neuronal degeneration in many systems (Simon *et al.*, 1984; Wieloch, 1985; Weiss *et al.*, 1986; Goldberg and Choi, 1993). It is possible that the neuroprotective effects of 4C3HPG may incorporate beneficial effects of mGluR 1/5 blockade together with beneficial effects of mGluR 2/3 activation; indeed, such combined activity may be a highly desirable characteristic for the future development of other candidate neuroprotective agents for the treatment of acute brain injury. In other studies, we (Buisson *et al.*, 1994), as well as Bruno *et al.* (1994), have found that the structurally different selective mGluR 2/3 agonist, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), also reduces NMDA toxicity, although generally to a lesser extent than achievable with 4C3HPG (unpublished observations).

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Exhibit AB (10/644,645)

Rapid Communication

The Metabotropic Glutamate Receptor Antagonist (+)-α-Methyl-4-carboxyphenylglycine Protects Hippocampal CA1 Neurons of the Rat from *In Vitro* Hypoxia/Hypoglycemia

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(Accepted 8 March 1994)

Summary—The ability of the selective metabotropic glutamate receptor (mGluR) antagonist α-methyl-4-carboxyphenylglycine (MCPG) to protect hippocampal CA1 neurons from a hypoxic/hypoglycemic injury was examined. Rat hippocampal slices were exposed to a 4 min hypoxia/hypoglycemia, and the recovery of evoked population spikes was monitored. 500 μM (+)-MCPG exhibited a statistically significant protective effect, whereas the (−)-isomer was ineffective. These data suggest that MCPG-sensitive mGluRs may contribute to hypoxia/hypoglycemia-induced injury in rat hippocampal slices.

Keywords—Hippocampus, hypoxia, hypoglycemia, metabotropic glutamate receptor, intracellular signal transduction.

Although several subtypes of glutamate receptors are functionally and pharmacologically characterized, initial studies concerned with glutamate neurotoxicity or hypoxia/ischemia were focused on the *N*-methyl-D-aspartate (NMDA) subtype. The idea that glutamate neurotoxicity is mainly mediated via this receptor is very attractive since the NMDA receptor gates an ion channel permeable to calcium. Calcium overload and loss of intracellular calcium homeostasis are thought to be crucial mechanisms leading to neuronal death (Hartley *et al.*, 1993).

However, non-ionotropic glutamate receptors may also play a role. At least two subtypes of the metabotropic glutamate receptor (mGluR) family, viz. mGluR1 and mGluR5, are able to initiate calcium release from internal stores by phospholipase C activation and subsequent inositol 1,4,5-trisphosphate formation (Tanabe *et al.*, 1992). The involvement of these receptors in mechanisms of neuronal damage has been suggested on the basis of the actions of the drugs L-aminophosphonopropionate (L-AP3) (Opitz and Reymann, 1991) and 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) in models of hypoxia (Opitz and Reymann, 1993) and neurotoxicity (Koh *et al.*, 1991). Furthermore it could be shown, that intracellular calcium stores contribute to the rise of $[Ca^{2+}]_i$ during hypoxia/hypoglycemia in hippocampal slices (Mitani *et al.*, 1993). However, the unavailability of selective and competitive antagonists made it difficult to determine if the activation of metabotropic glutamate receptors (mGluRs) takes part in posthypoxic or post-ischemic neuronal damage.

Recently, though, the (+)-isomer of α-methyl-4-carboxyphenylglycine (MCPG) has been found to be a selective and competitive mGluR antagonist with respect to ACPD-stimulated phosphoinositide hydrolysis, intracellular Ca^{2+}-mobilization, and ACPD-induced electrophysiological responses in rat brain and spinal cord (Bashir *et al.*, 1993; Jane *et al.*, 1993). In the present study we used this novel tool for mGluR pharmacology to investigate, whether the blockade of mGluRs can protect hippocampal CA1 neurones from hypoxic/hypoglycemic injury.

Experiments were performed on 7–8 week old male Wistar rats. The animals were killed by a blow to the neck. After decapitation and dissection of the brain, transverse hippocampal slices (400 μm) were prepared with a tissue chopper and placed into a superfusion chamber. There they were maintained at 33°C with a constant Ringer perfusion (1 ml/min). The Ringer contained (in mM): NaCl 124, KCl 4.9, $MgSO_4$ 1.3, $CaCl_2$ 2,

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KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10. The surface was exposed to a moist atmosphere of carbogen (95% O_2/5% CO_2). A population spike (PS) was evoked by stimulation of the Schaffer collateral/commissural fibers by biphasic square wave pulses with a duration of 0.1 msec per half cycle via a stainless steel electrode and recorded with a glass microelectrode (1–4 MΩ) in the stratum pyramidale of the CA1 region. Test stimuli were adjusted to elicit a PS of about 60% of its maximum amplitude. The amplitude was evaluated by calculating the voltage difference between the negative peak and the positive one preceding it.

Hypoxia/hypoglycemia was induced by changing the carbogen atmosphere of the chamber to a gas mixture containing 95% N_2/5% CO_2. Additionally, the bath solution was changed to a ringer containing 10 mM mannitol instead of D-glucose. Re-establishment of normal oxygen and glucose supply started 4 min after the onset of the insult. The electrophysiological recovery of PS amplitude was measured for 1 hr (the responses to four test stimuli with a frequency of 0.2 Hz were averaged). MCPG (Tocris Neuramin, Bristol, U.K.) was bath applied 20 min before hypoxia/hypoglycemia and washed out 10 min after reoxygenation.

Interruption of oxygen- and glucose-supply of hippocampal slices led to a total loss of the evoked electrophysiological response 1–2 min after the onset of the hypoxia/hypoglycemia. Following a 4 min hypoxia/hypoglycemia, the PS recorded from untreated CA1 pyramidal cells recovered within 1 hr only to about 50% of its baseline amplitude (Fig. 1). Thereafter the degree of recovery remained constant for the next 60 min (data not shown).

If 500 μM (+)-MCPG was present during hypoxia/hypoglycemia, PS responses recovered to approximately 75% ($n = 6$), significantly different from the recovery in control slices (Mann–Whitney U-test, $P < 0.05$). This protective effect was not observed, when the (−)-isomer of this mGluR antagonist was applied ($n = 9$; Fig. 1). Neither (+)- not (−)-MCPG affected the baseline responses (data not shown).

We investigated in the present study the role of a possible activation of mGluRs in an *in vitro* model of hypoxic/hypoglycemic injury. In this model, a 4 min period of hypoxia/hypoglycemia led to a disturbance of the ability of CA1 neurones to respond to an activation of a synaptic input with the generation of a PS. The amplitude of the PS depends, over a wide range, on the number of discharging neurones (Anderson *et al.*, 1971), and may therefore serve as a standard of functional integrity of this cell population.

The attenuation of this functional disturbance by the selective and competitive mGluR antagonist (+)-MCPG is in accordance with our previous finding, that L-AP3 is able to protect CA1 neurones from hypoxic injury *in vitro* (Opitz and Reymann, 1991). Since MCPG does not affect ionotropic glutamate receptors (Bashir *et al.*, 1993), we suppose that mGluRs are the site of action in our preparation and that activation of mGluRs could play a role in mechanisms leading to post-hypoxic/hypoglycemic damage. However, this seems to be in contrast to other reports indicating that the mGluR agonist ACPD is able to attenuate NMDA neurotoxicity (Koh *et al.*, 1991) as well as hypoxic injury (Opitz and Reymann, 1993). There are at least two possible explanations for this contradiction.

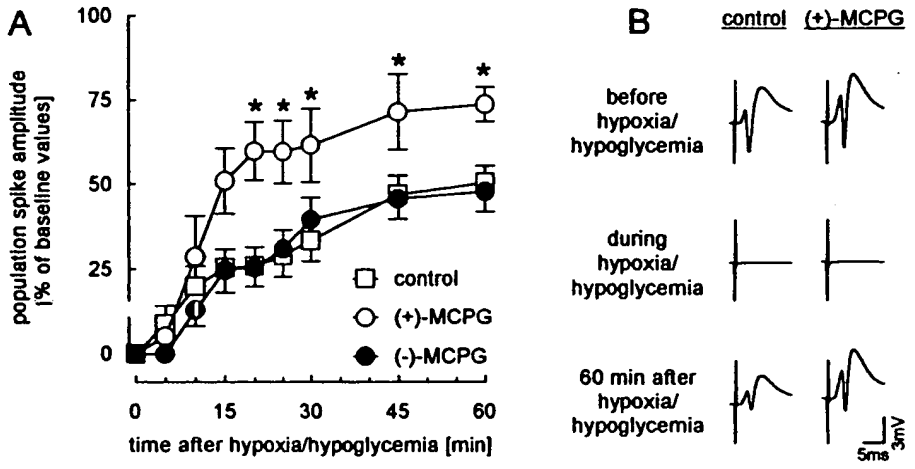

Fig. 1. Effect of (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) on the recovery of population spikes in area CA1 of rat hippocampal slices, which underwent a 4 min hypoxia/hypoglycemia. Time course of population spike recovery in untreated control slices (□, $n = 12$) and slices treated with either 500 μM (+)-MCPG (○, $n = 6$) or 500 μM (−)-MCPG (●, $n = 9$) as described in the text. The stars indicate statistically significant differences to the control slices, calculated with Mann–Whitney's U-test ($P < 0.05$). (B) The representative traces show evoked population spikes recorded immediately before the onset of hypoxia/hypoglycemia (upper row), during (middle row), and 1 hr after the insult (lower row).

(i) There is a massive glutamate release during hypoxia/hypoglycemia (Mitani *et al.*, 1991). With such a high glutamate concentration, ACPD, L-AP3, and MCPG could exhibit other pharmacological effects (e.g. as a partial agonist) than those described in experimental conditions with "normal" oxygen- and glucose-supply.

(ii) A more likely hypothesis is that ACPD and the mGluR antagonists exhibit their protective action via different receptor subtypes and subsequently via different intracellular pathways.

In conclusion, using the specific and competitive antagonist MCPG, we have shown that activation of mGluRs might play a role in the mechanisms related to neuronal injury during hypoxia/hypoglycemia. Since (+)-MCPG is an effective antagonist at the phospholipase C-coupled mGluR1 (Bashir *et al.*, 1993) this suggests that at least this subtype might be important in hypoxia/hypoglycemia.

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Exhibit AC (10/644,645)

Plasma amino acid levels in patients with amyotrophic lateral sclerosis

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Key words: Amyotrophic lateral sclerosis; Amino acid; Aspartate; Glutamate; Glycine

Summary

Evidence for a generalized defect in glutamate in patients with amyotrophic lateral sclerosis (ALS), associated with widespread alterations in the central nervous system level of this excitatory amino acid. We measured fasting plasma amino acid in 10 ALS patients and 10 controls matched for age and sex. ALS patients had statistically significant elevations in plasma level of aspartate, glutamate, and glycine. The plasma levels of other amino acids were not significantly different from those found in controls. No correlation between ALS severity or activity and degree of abnormality in amino acids was established.

Introduction

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurological disorder that is manifested clinically by muscle weakness, wasting, and spasticity and pathologically by degeneration and loss of the motor neurons in the spinal cord, brainstem, and cerebral cortex. The cause of this disease is still unknown (Mitsumoto et al. 1988; Iwasaki et al. 1989). Little attention has been paid to possible abnormalities of amino acid (AA) neurotransmitters in the central nervous system (CNS) of ALS patients and recent studies showed a systemic defect in glutamate metabolism in ALS (Patten et al. 1978; Plaitakis and Caroscio 1987; Perry et al. 1990; Rothsteinet et al. 1990). Glutamate is thought to serve as the major excitatory neurotransmitter of the corticospinal pathway, which is remarkably involved in ALS (Young et al. 1983). Of particular interest, excitatory neurotoxins derived from the ingestion of cycad nuts may, in part, be responsible for the motor neuron degeneration associated with the ALS/parkinsonism-dementia complex of the Chamorro population of the Mariana islands (Spencer 1987). Therefore, we decided to do a study of plasma-free AA in 10 patients with ALS and to compare the levels with those found in a control population matched for age and sex.

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Materials and methods

Patients and control subjects

Plasma was obtained after an overnight fast, from 10 patients with ALS (age range 21–69 years; mean ± standard deviation (SD), 55 ± 15 years). There were 6 men and 4 women. All patients were examined by one or more experienced neurologists, and all met the following diagnosis criteria: (1) a combination of upper and lower neuron signs, or only lower motor neuron signs, commencing focally and showing a progressive asymmetrical spread; (2) absence of sensory symptoms and signs; (3) normal findings on computed tomography, myelography, or magnetic resonance imaging studies; (4) no evidence of multiconduction block on nerve conduction studies; (5) electromyographic evidence of acute and chronic denervation of involving limb and axial musculature; and (6) no evidence of heavy metal intoxication, monoclonal gammopathy, or endocrine abnormalities. Ten healthy adults served as normal controls. All subjects were free of any medications for at least 7 days when studied, and were not known to suffer from psychiatric disease, thyroid disease, diabetes, epilepsy, cardiovascular disease, or coagulopathy. There were no differences in diets between ALS and controls.

Clinical indices of patients

Severity of disability was rated from 1 to 4 at the time the sample for AA analysis were obtained; 1 = little or no interference with work and daily activity; 2 = moderate interference with work and daily activities but able to work and able to earn a living for self; 3 = severe interference with daily activities, unable to work but able to do some things for self; 4 = totally dependent on others for care (Patten et al. 1978). Severity of illness ranged from 1 to 4 in ALS patients.

In ALS patients the duration of illness was taken as the time interval in years between the time of examination and the time patient thought he noticed the first symptom. Duration of illness in ALS varied from 0.3 to 2.3 years with a mean of 1.1 years. The activity index was derived for each ALS patient by dividing the severity index by the duration of illness in years. For instance, a patient with a severity score of 2 whose illness had lasted 2.3 years would have an activity index of 0.9. The range of activity indices in the ALS group was 0.9 to 13.3 with a mean of 4.0.

Biochemical methods

Fasting blood specimens were collected in heparinized tubes and were immediately centrifuged at $20\,000 \times g$ for 10 min. The supernatant plasma was removed with a Pasteur pipette, taking care not to aspirate any of the buffy coat. Plasma was then deproteinized within 30 min of venipuncture by adding 30 mg solid sulfosalicylic acid per ml, shaking to mix, and centrifuging at $20\,000 \times g$ for 10 min. Deproteinized plasma supernatants were stored at −70°C until analyzed (Perry et al. 1990). Analysis of plasma AA was performed by automated ion-exchange chromatography with lithium-based buffer and a Beckman 6300 AA analyzer (Beckman Instruments, Fullerton, CA). Evaluation of ALS and control samples was done in parallel in the same assay.

Results

Table 1 summarizes the data on clinical characteristics of our ALS patients. Table 2 lists the mean concentration of numerous AA and related compounds in fasting plasma of control subjects and patients with ALS. Mean concentrations of aspartic acid, glutamic acid, and glycine were significantly increased in the plasma of the patients with ALS. The plasma levels of other AA were not significantly different from those found in controls. We also examined whether there was any relationship between plasma AA levels and severity, duration, and activity in ALS patients, but there was no relationship between them.

Discussion

The protean pathological changes of ALS include selective atrophy of the motor neurons in the spinal cord and brainstem usually occurring with degeneration of the corticospinal tracts and pyramidal neurons of the motor cortex. However, in some patients more widespread neurohistological changes are found (Tandan and Bradley 1985a,b). In view of these rather widespread pathological changes, it seems logical to consider that a generalized metabolic defect may underline the disease (Patten et al. 1987). Our study showed that concentrations of the AA aspartate, glutamate, and glycine are selectively elevated in the plasma

TABLE 1

CLINICAL CHARACTERISTICS OF ALS PATIENTS

Pat.	Sex	Age (yrs)	Duration (yrs)	Severity index [a]	Activity index [b]	Clinical form	Bulbar signs [c]	Upper motor signs [d]	Lower motor signs [e]
1	F	21	2.3	2	0.9	ALS	+	+	+
2	M	69	0.3	2	6.6	ALS	+	+	+
3	F	66	0.3	4	13.3	ALS	+	+	+
4	M	40	1.3	4	3.1	ALS	+	+	+
5	M	56	0.5	2	4.0	ALS	+	+	+
6	M	67	2.0	4	2.0	ASL	+	+	+
7	F	62	1.5	3	2.0	ALS	+	+	+
8	F	53	1.3	2	1.5	ALS	+	+	+
9	M	56	0.6	3	5.0	ALS	+	+	+
10	M	60	1.0	2	2.0	ALS	+	+	+
Mean ± SD		55.0 ± 14.6	1.1 ± 0.7	2.8 ± 0.9	4.0 ± 3.7				

[a] Severity index was determined as indicated in the text.
[b] Activity index was determined as indicated in the text.
[c] Bulbar signs comprise tongue paralysis, dysphagia, and dysphonia.
[d] Upper motor signs include Babinski sign, brisk tendon reflexes, and spasticity.
[e] Lower motor signs include amyotrophy, fasciculation, and muscle weakness.

TABLE 2

CONCENTRATIONS OF AMINO ACIDS AND RELATED COMPOUNDS IN FASTING PLASMA OF ADULT CONTROL SUBJECTS AND PATIENTS WITH ALS

Amino acid compound	Plasma [a]	
	Normal control subjects ($n = 10$)	Patients with ALS ($n = 10$)
Taurine	60.5 ± 6.6	74.6 ± 28.5
Phosphoethanolamine	(Tr.–1)	(Tr.–1)
Aspartate	2.4 ± 1.1	6.0 ± 5.2 [b]
Hydroxyproline	(0–1)	(0–23)
Threonine	151.8 ± 29.3	129.8 ± 32.7
Serine	120.2 ± 22.2	123.1 ± 49.3
Asparagine	51.9 ± 8.2	61.6 ± 17.5
Glutamate	34.1 ± 11.3	163.9 ± 117.3 [d]
Glutamine	568.4 ± 90.1	571.5 ± 257.9
Proline	204.6 ± 39.0	172.4 ± 53.5
Glycine	225.0 ± 43.9	311.1 ± 95.9 [c]
Alanine	400.9 ± 79.0	338.5 ± 186.3
Citrulline	34.5 ± 6.3	31.9 ± 13.7
α-Amino-*n*-butyric acid	(0–1)	(0–1)
Valine	250.2 ± 126.1	261.1 ± 75.2
Cystine	39.4 ± 9.6	33.3 ± 20.2
Methionine	31.2 ± 4.8	33.4 ± 5.2
Isoleucine	75.7 ± 12.3	70.0 ± 23.6
Leucine	125.7 ± 18.4	104.2 ± 31.3
Tyrosine	62.9 ± 5.8	54.3 ± 29.7
Phenylalanine	64.9 ± 10.3	56.1 ± 43.4
Tryptophan	55.1 ± 12.1	37.9 ± 18.9
Ornithine	109.8 ± 12.7	109.2 ± 40.2
Lysine	81.2 ± 16.6	105.0 ± 113.8
Histidine	53.0 ± 15.6	54.5 ± 38.4
1-Methylhistidine	(0–Tr.)	(0–4)
3-Methylhistidine	32.8 ± 30.7	33.6 ± 4.2
Arginine	(0–1)	(0–1)

[a] Values (means ± SD) are expressed as μmol/l. Ranges are shown in parentheses. Significance listed is difference from control subjects.
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] $P < 0.001$.
Tr., trace.

of patients with ALS. Levels of free AA have previously been studied in patients with ALS and abnormalities in plasma levels of several AA have been described (de Belleroche et al. 1984; Plaitakis and Caroscio 1987). In contrast to the results of our study, however, no significant changes in the fasting plasma levels of glutamate were noted by Patten and associates (1978). Perry et al. (1990) also reported that glutamate concentrations were normal in the fasting plasma of the patients with ALS. Laboratory techniques play an important role in the determination of AA, especially glutamate and aspartate (Lau et al. 1990; Spink and Martin 1991). If blood is not centrifuged at a sufficiently high speed (21 000 × *g* in study by Perry et al.; 20 000 × *g* in our study; 19 000 × *g* in study by Plaitakis and Caroscio), artifactual elevation in the levels of glutamate can occur and also, if deproteinized plasma is left at room temperature, artifactual elevation in the levels of glutamate will occur (Plaitakis et al. 1982; Plaitakis and Caroscio 1987). It is considered that there were no problems concerning laboratory techniques in our procedure for the determination of AA. An important factor that could enhance glutamatergic transmission is innervation by interneurons, as has been shown in cultured chick motor neurons (O'Brien and Fischbach 1986). A substantial proportion of such spinal cord interneurons, particularly those forming synapses in the ventral horns, are thought to be glycinergic (Price et al. 1976). Glycine has been recently shown to potentiate glutamatergic transmission by acting on a strychnine-insensitive allosteric site of the *N*-methyl-D-aspartate (NMDA) receptor (Johnson and Ascher 1987). Glycine seems to increase the frequency of the NMDA receptor channel opening, probably by accelerating the recovery of the receptor from glutamate-induced desensitization (Mayer et al. 1989; Plaitakis 1990; Young 1990). Considering the free AA levels in the plasma of patients with ALS, Patten et al. (1978) noted that the clinical activity of ALS correlated directly with plasma aspartate concentrations, but there was no relationship between plasma AA levels and clinical characteristics in our study. The neurotoxicity of endogenous excitatory AA might be involved in neurodegenerative disorders (Coyle 1982) or mediate neuronal damage in response to various insults (Wieloch 1985; Rothman and Olney 1986). In view of these considerations, the present data, showing that plasma AA aspartate, glutamate and glycine are altered in ALS, raise the possibility that neuroexcitotoxic mechanisms may be involved in the neurodegeneration of ALS.

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